Identifying the autoantigen of a diabetogenic CD8 T cell clone isolated from young NOD mice.

Caroline R. Dumont

Yale University

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Caroline R. Dumont

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Abstract

Defining the autoantigenic targets of T cells involved in insulin-dependent diabetes mellitus (IDDM) is essential for understanding the pathogenesis of diabetes. IDDM results from the autoimmune destruction of the pancreatic islet β cells. The non-obese-diabetic (NOD) mouse is a murine model for spontaneous IDDM. T cells play an important role in the pathogenesis of diabetes, and CD8 T lymphocytes may be critical initiators of diabetes. Several β-cell specific autoantigens of CD4 T cells have been identified. However, the autoantigens of CD8 T cells, which are likely to play an important role in the induction of diabetes, have not yet been identified. In this thesis we describe the application of a single-cell T cell assay, which takes advantage of a *lacZ* reporter construct, as a means to identify the target antigen of a diabetogenic CD8 T cell clone isolated from young NOD mice. Indicator cells are *lacZ* -inducible T-cell hybrids specific for the unknown antigen, and are shown here to turn blue in the presence of NOD mouse islets and the chromogenic substrate X-gal. These T cell hybrids, and COS-7 cells transfected with the murine K^d MHC class I molecule as antigen-presenting cells, have been used to screen a NOD mouse islet cDNA library for the target antigen of the diabetogenic CD8 T cell clone. Our findings suggest that increasing the sensitivity and specificity of the *lacZ* assay may be necessary for detecting autoantigenic cDNA when it exists at very low frequencies in the cDNA library.

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Introduction

Overview

Identifying the antigens on β cells targeted in type 1 diabetes is key to understanding the pathogenesis of this autoimmune disease, and promises to be useful for early diagnosis, and specific modulation of the immune process. Many researchers have searched for the antigen responsible for inducing diabetes in non-obese diabetic (NOD) mice. Their results have been enticing, yet remain controversial, and the existence of a dominant disease-initiating antigen has not been identified. Most of the candidate antigens proposed thus far are recognized by CD4 T cells, and not CD8 T cells, which may be the critical initiators of diabetes. Here we present the application of a recently introduced method that sensitively measures T-cell activation, and a strategy for identifying class I-restricted T cell antigens (Karttunen et al., 1992), as a means to elucidate the target antigen of cloned CD8 T cells (Wong et al., 1996) which were obtained at an early stage of disease in the NOD mouse, and which cause rapid-onset diabetes in the absence of CD4 T cells.

1. T cell maturation: MHC restriction and self tolerance

A critical function of T cells is to distinguish healthy tissue from infected tissue. They do so by recognizing foreign or altered proteins on the surface of cells in the context of MHC molecules. There are two classes of MHC molecules which differ in the source of the peptides they bind and carry to the cell surface. MHC class I molecules bind fragments of endogenous peptides, such as self antigens and fragments of viral proteins, generated in the cytosol and then transported to the endoplasmic reticulum (Figure 1) (Germain and Margulies, 1993). Extracellular antigenic proteins such as from

most bacteria are made visible to the immune system via the MHC class II presentation pathway. Class II molecules sample the contents of intracellular vesicles, and thus display peptide fragments which have been internalized by phagocytic cells which include macrophages, B cells, and specialized antigen processing cells (APCs) called dendritic cells (Figure 1) (Germain and Margulies, 1993).

T cell precursors arise in the bone marrow and travel to the thymus (Ikuta et al., 1992). Here, gene rearrangements results in the assembly and expression of unique, exclusively membrane-bound T cell receptors (TCRs) (Matis, 1990). Subsequently, T cell maturation occurs and comprises two selective processes known as positive selection and negative selection.

Positive selection ensures that T cells recognize self MHC molecules. All T cell receptors recognize MHC genes of the species, and virtually all of them recognize antigen in the form of peptide:MHC complexes. The ability of T cells to recognize specific antigens only in the context of self MHC molecules is also called MHC restriction. In order to receive important developmental signals from the thymic microenvironment, T cells must still possess an affinity for self-MHC molecules. Furthermore, MHC restriction ensures that T cells can potentially bind these molecules when they contain foreign antigenic peptides (Hogquist et al., 1994). Through a process that is not well understood T cells that recognize peptides in the context of MHC class I molecules (class-I-restricted) mature into cytotoxic T cells, expressing the CD8 co-receptor, whereas thymocytes that recognized peptides in the context of MHC class II molecules (class-II-restricted) generally mature into helper CD4 T cells (Kaye et al., 1989; Kisielow et al., 1988; Sha et al., 1988). CD4 T cells differentiate into two functional types depending on their cytokine profiles: Th1 cells and Th2 cells. Cells that do not encounter their restricting

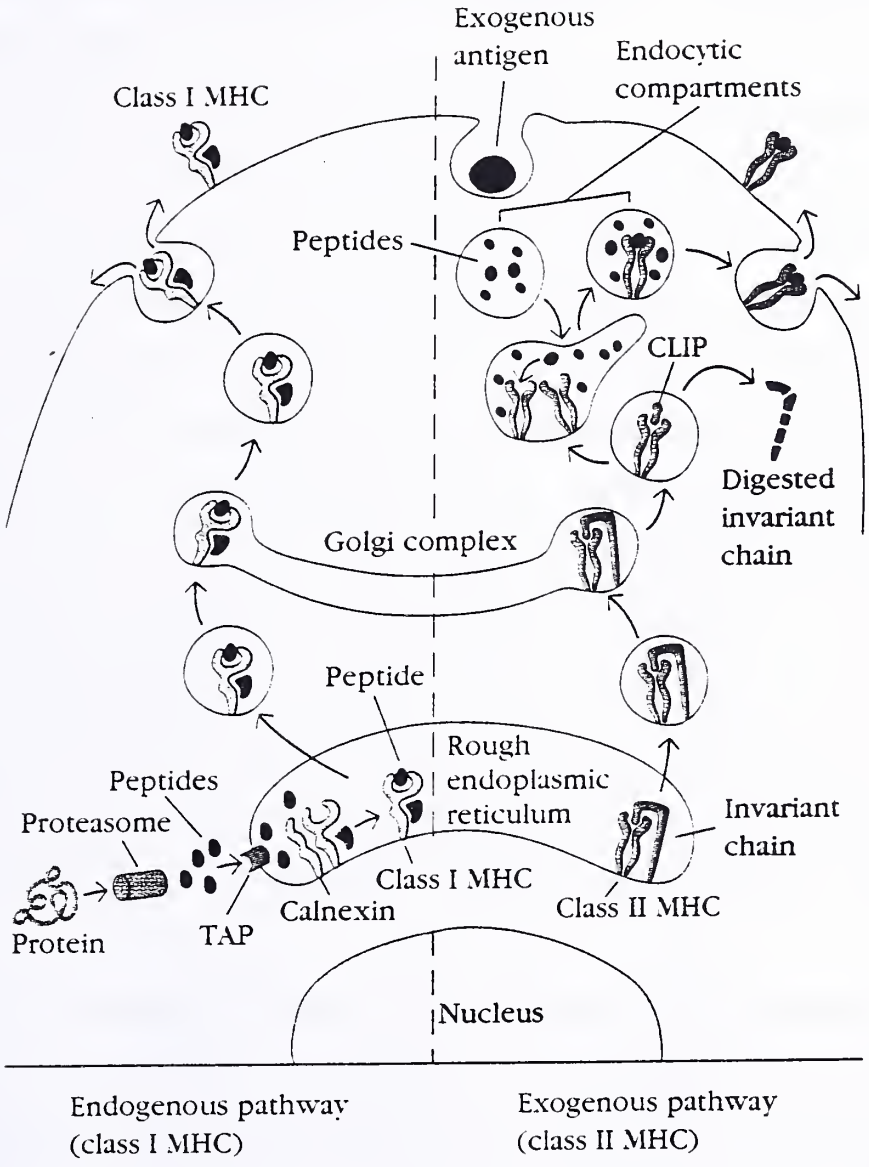


Figure 1. Schematic representation of the MHC class I and class II antigen processing pathways. Endogenous synthesis of protein antigens results in the production of peptide-MHC class I complexes. Peptide- MHC class II complexes are generated only in cells that express MHC class II molecules, such as professional APCs, via endocytosed native proteins or phagocytosed bacteria. [Figure adapted from Kuby, J. (1997) *Immunology* 3rd ed (W.H. Freeman and Company. New York, New York) p. 257]

MHC molecule in the thymus die before attaining functional maturity (Surh and Sprent, 1994).

Negative selection ensures that T cells are not reactive to MHC-peptide ligands found on self tissue, and are thus self-tolerant (von Boehmer et al., 1993). Many thymocytes that are positively selected in the thymus possess receptors which are reactive to self peptides (Pullen et al., 1988; Sha et al., 1988). These self-reactive thymocytes are eliminated either through programmed cell death in the thymus, also called clonal deletion, or remain functionally inactive in the periphery (Miller and Morahan, 1992).

Only the thymocytes whose TCRs have met the two selection criteria: self-tolerance and self-restriction, develop into mature thymocytes (Sha et al., 1988). This functions as a stringent screen mechanism upon developing thymocytes. In fact it is estimated that during negative and positive selection the vast majority of thymocytes, estimated around 98%, never leave the thymus but undergo apoptosis (Strasser, 1995; Surh and Sprent, 1994).

2. Co-stimulatory molecules and peripheral T cell tolerance

T cell activation is a complex process that requires at least two simultaneous signals from the same professional APC. The first is delivered through the T cell receptor (TCR), and involves recognition of a peptide fragment of antigen presented in the context of MHC molecules on the surface of an APC. The second signal, referred to as co-stimulation, is mediated through the binding of CD28 on T cells to co-stimulatory molecules on the same APC. Costimulation is required for optimal activation of naive T cells (Liu and Janeway, 1992). In fact, rather than activation, engagement of the TCR complex of naive T cells in the absence of co-stimulation leads to T cell anergy (Gimmi et al., 1993; Schwartz, 1990) or cell death (Liu and Janeway,

1992). It is thought that the requirement of two signals by one cell represents a mechanism for maintaining self-tolerance. Self tolerance would be lost if T cells recognizing self-antigens on the surface of tissue cells could be co-stimulated by resident or distant APCs, and not by the cell bearing the specific antigen (Schwartz, 1989).

The best characterized costimulatory molecule, B7-1, interacts with receptors on the surface of T cells. These include CD28 and CTLA-4, a receptor which is expressed only once T cells are activated and for which B7-1 has a very high affinity to (Freeman et al., 1991; Linsley et al., 1991). B7-CD28 interaction has been clearly shown to activate CD4 T cells, and leads to the production of cytokines such as IL-2 and IFN-γ (Freeman et al., 1991; Gimmi et al., 1993; Linsley et al., 1991). The role of the costimulatory pathway for CD8 T cells is less clear, although it has been demonstrated that these cells can also be activated following B7-1 interaction (Tan et al., 1992). However, it seems that B7-1 interaction may stimulate T cell activation and not effector function (Harding and Allison, 1993).

3. Autoimmunity: loss of self-tolerance

Autoimmune disease occurs when a specific adaptive immune response is mounted against self antigens, or when self-tolerance is lost. Since negative selection, and peripheral inactivation by the absence of co-stimulation, are both very efficient processes it is unlikely that autoimmunity results from the failure of these two major mechanism of self tolerance (Janeway and Travers, 1997). Antigens that are expressed abundantly in the periphery induce clonal deletion or anergy. On the other hand, very rare antigens that are presented poorly on tissue cells are unable to activate antigen-specific T cells, which are present in the individual. These cells are

not normally activated, and are said to be immunologically ignorant. They require special circumstances to be activated and are likely responsible for most autoimmunity. Antigens which are presented at intermediate levels on tissue cells, such as those selectively expressed in the thyroid or the pancreatic islets, usually do not activate immunologically ignorant T cells, since the antigen would not be presented along with a co-stimulatory molecule. However, if the T cell was inappropriately activated, it would then go on to attack such tissues (Janeway and Travers, 1997; Katz et al., 1993).

Another theory of the mechanisms underlying the loss of self-tolerance involves a change in the balanced state between aggressive and protective immune mechanisms. In both the human disease and the NOD mouse model, diabetes is preceded by a long phase of islet-cell-specific autoreactivity, before hyperglycemia is manifested, and this silent phase is thought to correspond to the active suppression of autoreactive T cells by regulatory, or suppressor T cells, in the periphery. In NOD mice, transfer of certain islet-specific T cell clones can prevent the destruction of pancreatic islet cells (Reich et al., 1989). Likewise, during the early phase of insulitis mice are resistant to the transfer of disease by diabetogenic T cells (Bendelac et al., 1987), and this resistance can be broken down by selectively depleting CD4 T cells (Sempe et al., 1994). It is unclear what triggers the breakdown from active tolerance to overt diabetes. However, it is thought that self-tolerance to pancreatic β cells is maintained by CD4 regulatory Th2 T cells, that produce IL-4 and IL-10, and provide help for the activation of B cell-mediated humoral responses. Th2 regulatory cells seem to suppress the activation of CD4 autoreative Th1 cells, that produce INF-γ, and IL-2, and tend to activate macrophages and cytotoxic CD8 T cells (Cameron et al., 1998; Pilstrom et al., 1995; Rabinovitch et al., 1995). There is evidence that when diabetes occurs

there is a shift to a Th1 profile (Shimada et al., 1996), and that protection can be brought out from a shift from a Th1 to a Th2 type of response (Cameron et al., 1997; Mueller et al., 1996). However, it is likely that a number of immune defects in addition to a Th1/Th2 imbalance contribute to the development of diabetes in the NOD mouse. Furthermore, the relevance of such deficiencies in NOD mice has yet to be correlated to human disease.

4 The NOD mouse

Overview

Type 1 diabetes results from the interplay of genetic, environmental, and autoimmune factors, and leads to the selective destruction of insulin-producing pancreatic β cells. NOD mouse diabetes develops spontaneously, and possesses striking similarities to human type 1 diabetes. As such, the NOD mouse has become the most widely studied model of human diabetes. NOD mouse diabetes is not completely identical to human diabetes. For instance, there is no gender predominance in human diabetes whereas in NOD mice there is. In addition, high levels of islet-reactive autoantibodies exist in humans, whereas only low levels are found in NOD mice. Nevertheless, the NOD mouse model may serve to uncover the pathogenesis of diabetes, and allows for insight into the development of autoimmune diabetes in humans.

The NOD mouse was described as a diabetes model in Japan in the late 1970s (Makino et al., 1980). The mice develop an inflammatory infiltrate, called "insulitis", within the pancreatic islets at about 3-4 weeks of age, or the time of weaning. Dendritic cells and macrophages perhaps infiltrate first (Jansen et al., 1994), followed by T cells (both CD4 and CD8) and B cells (Kay et al., 1991; O'Reilly et al., 1994). Diabetes begins to occur at about 12 weeks of

age, correlating with islet β cell destruction, and hyperglycemia (Bach, 1994). In addition, the appearance of autoantibodies (Bottazzo et al., 1989; Castano and Eisenbarth, 1990), defects in T cell activity (Serreze and Leiter, 1994), the sensitivity of the disease to immunosuppression (Bottazzo et al., 1989), and the presence of predisposing major histocompatibitiy complex (MHC) genes (Wicker et al., 1995) all suggest an autoimmune basis for the disease.

A. Idd genes and MHC class II molecules

IDDM in the NOD mouse is controlled by multiple susceptibility (Idd) genes both within and outside the MHC (Serreze and Leiter, 1994). As many as 15 loci critical to the IDDM process have been detected by genetic studies (Todd et al., 1991), though only a very few have been identified.

The most important genetic component leading to IDDM susceptibility in NOD mice is the strain's MHC genes (Vyse and Todd, 1996). The MHC class II region, formerly *Idd1*, has been identified as a necessary but not sufficient predisposing genetic factor for diabetes (Tisch and McDevitt, 1996). The NOD mouse MHC class II molecule, I-A^{g7}, the homologue to human leukocyte antigen (HLA) DQ molecules, is composed of an I-$A\alpha^{d}$ chain plus a unique I-$A\beta^{g7}$ chain that contains histidine-serine residues at amino acid positions 56 and 57, rather than the usual proline-aspartic acid residues seen in most other strains (Acha-Orbea and McDevitt, 1987). Diabetes can be prevented when I-A^{g7} is altered by substitution at position 56 (Lund et al., 1990). The I-A^{g7} motif is remarkably similar to HLA DQ8, in that linkage between the lack of aspartic acid at the 57 position of the β chain and susceptibility to diabetes is also found with human DQ molecules (Kwok et al., 1996; Todd et al., 1988).

The nature of the binding properties of the I-A^{g7} allele has been studied, but remains controversial. Work by Cohen and colleagues suggests that the peptide-binding motif of I-A^{g7} is similar to that of HLA DQ (Reizis et al., 1997; Reizis et al., 1997). Their findings suggest that the I-A^{g7} molecule binds peptide adequately, and that it induces good T cell responses. Harrison et al (Harrison et al., 1997) propose a different I-A^{g7} peptide-binding motif, though they also find that the I-A^{g7} molecule is a good peptide-binder. On the other hand, others have found that I-A^{g7} molecules are unstable, and are poor peptide binders (Carrasco-Marin et al., 1997). This has led to the hypothesis that, since elimination of autoreactive T cells requires a threshold period of time in contact with peptide-MHC complexes (Ashton-Rickardt and Tonegawa, 1994), T cells in contact with unstable complexes would tend to escape negative selection in the thymus. In fact, studies have found that mice carrying the H-2^{g7} class II molecules contained about 8-fold more autoreactive T cells, than their congenic counterparts bearing H-2^{b} MHC (Kanagawa et al., 1998). This issue remains to be resolved and has important implications not only for T cell selection but T cell reactivity in response to antigen presentation. The diabetogenicity of the NOD mouse $H2^{g7}$ MHC haplotype is further enhanced by the lack of I-E expression on NOD APC's, due to a mutation in the first exon of the Eα gene (Ikegami et al., 1990). IDDM is inhibited in the NOD mouse with expression of the I-E transgene derived from diabetes resistant MHC haplotypes (Lund et al., 1990; Nishimoto et al., 1987).

B. MHC Class I molecules

Though the role of MHC class I region has been studied less than that of class II, it is clear that common class I alleles, such as K^{d} or D^{b}, encoded by

the H2g^7 MHC haplotype of NOD mice play an essential role in the development of IDDM. As it will be described later, the requirement of MHC class I expression was demonstrated following the development of β2-microglobulin knock-out (β2m^{null}) NOD mice which do not express H-2g^7 class I molecules, and remain free of diabetes (Serreze et al., 1994). IDDM is also inhibited in NOD mice that are congenic for the MHC haplotype of the CTS mouse strain, that shares the same MHC class II allele, but not the class I allele, of the NOD mouse (Ikegami et al., 1995). Similarly, it has been recently demonstrated that certain MHC class I alleles such as HLA-A2 in humans may play a role in enhancing IDDM susceptibility (Demaine et al., 1995; Fennessy et al., 1994).

5. The diabetogenic roles of CD4 and CD8 T cells

Overview

Diabetes is an ancient disease and is quite prevalent, yet facets of this disease are poorly understood, including the immunological mechanisms which initiate it. It has been established that IDDM results from a T-cell dependent autoimmune process directed against the pancreatic β cells, however the role of these particular T lymphocyte subsets, and their target antigens, remains to be elucidated.

T cells are important in the pathogenesis of diabetes. Histological analysis of islet lesions reveal the presence of both CD4 and CD8 T cells (Miyazaki et al., 1985). Injection of anti-CD3 antibodies inhibits disease progression (Hayward and Shreiber, 1989). However, the precise contribution of CD4 and CD8 T cells to disease progression is uncertain. Several groups have shown that both CD4$^+$ and CD8$^+$ T cells from young diabetic donors are required for disease transfer into neonatal, irradiated young, or genetically T

cell-deficient NOD recipients (Bendelac et al., 1987; Christianson et al., 1993; Miller et al., 1988; Rohane et al., 1995) indicating that these cells likely have synergistic roles. CD4 T cells alone invade the islets, but diabetes develops only in the presence of CD8 T cells. CD8 cells, alone, cannot transfer insulitis or diabetes (Christianson et al., 1993; Thivolet et al., 1991). These findings led to the notion that CD4 T cells may initiate diabetes, and recruit CD8 T cells, the effector cells capable of carrying out the final destruction of islet β cells. This idea was consistent with other data, such as the isolation of CD8 T cell clones with *in vitro* and *in vivo* anti-islet beta cell cytotoxic activity [Reich et al, 1989 #40; Nagata, 1989 #474; Hayakawa, 1991 #290; Nagata, 1994 #239; Nagata, 1992 #242]. Some studies found that CD4 cells precede the entry of CD8 T cells into islets, following the transfer of whole splenocytes from diabetic donors into to healthy recipients (O'Reilly et al., 1991). On the other hand, others have shown that macrophages and CD8 T cells are the earliest infiltrating cells (Bedossa et al., 1989; Jarpe et al., 1990).

A. CD8 T cells as initiators of diabetes

More recently, there is an increasing body of evidence that CD8 T cells are important in the initiation of diabetes in addition to playing a cytotoxic role in the final stages of β cell destruction. Pancreatic β cells are generally not regarded as effective APC's, possessing MHC class I molecules, but not class II molecules on their surface (McInerney et al., 1991; Signore et al., 1989). Therefore, if CD4 T cells are to recognize β cell antigens, they must do so in the context of I-A^{g7} molecules on the surface of professional antigen-presenting cells (APC's), such as macrophages and dendritic cells residing in the pancreatic islets. As such, it seems likely that an initial β cell insult occurs, mediated by MHC class I-restricted CD8 T cells, followed by the release

of soluble antigens that are then processed and presented by APCs to CD4 T cells. An initiating function of CD8 cells would also be more in line with human diabetes, where CD8 T cells seem to dominate the islet infiltrate of in newly diagnosed patients (Bottazzo et al., 1985; Hanninen et al., 1992; Itoh et al., 1993) and are highly activated in the blood (Hehmke et al., 1995).

B. MHC Class I molecules and the role of CD8 T cells

The strongest evidence that CD8 T cells may be important in the initiation of type 1 diabetes comes from studies in which NOD mice are bred to lack functional MHC class I molecules and consequently most CD8 T cells. MHC class I molecules consist of a 45 kD polypeptide chain, the alpha chain, which is non-covalently associated at the cell surface with β2-microglobulin (β2-m). In 1990 it was reported by two groups that following the disruption of the β2-m gene by targeted mutation, mice are unable to express β2-m, and therefore MHC class I molecules (Koller et al., 1990; Zijlstra et al., 1990). However, MHC class II expression and CD4 cells appears normal. Consequently, these mice lack virtually all mature CD8 T cells, supporting previous evidence that interactions with class I MHC molecules are essential for CD8 T cell development (Sha et al., 1988; Zuniga-Pflucker et al., 1989).

As a means to further examine the relative contributions of MHC class I-restricted and MHC class II-restricted T cells to autoimmune pancreatic β cell destruction, several groups bred NOD mice with β2-m knock-out mice (NOD-β2m^{null}) (Katz et al., 1993; Serreze et al., 1994; Sumida et al., 1994; Wicker et al., 1994). It was found that NOD mice deficient in MHC class I expression and CD8 T cells, remained completely free of both insulitis and diabetes. These results seem to argue that CD8 T cells are crucial for the initiation of diabetes in NOD mice. Further evidence of the initiating role of CD8 T cells in

diabetes came from studies demonstrating the inhibition of insulitis following the injection of anti-CD8 monoclonal antibodies into young NOD mice, between 2 and 5 weeks of age (Wang et al., 1996). These results also suggest that it is the lack of CD8 T cells, rather than the lack of MHC class I molecules, which prevents infiltration of the islets. Finally, supporting evidence for the participation of CD8 T cells in disease initiation has come from studies demonstrating that T cells from young prediabetic NOD mice transfer disease to MHC class I-positive NOD-SCID mice but not to NOD-SCID mice bearing the $\beta 2m^{null}$ mutation (Serreze et al., 1997). It is also evident that the absence of CD4 T cells, such as with NOD.CD4-/- mice (Wong et al., 1998), and following anti-CD4 antibody treatment (Shizuru et al., 1988), prevents insulitis and diabetes. Thus, there is a dual role played by CD4 T cells and CD8 T cells in disease initiation.

It is important to recognize that the critical role of CD8 T cells in initiation of diabetes does not exclude their effector function. The transfer of diabetes to NOD-$\beta 2m^{null}$ recipients with diabetic spleen cells is considerably delayed compared with wild-type NOD mice (Kay et al., 1996; Wicker et al., 1994). It is possible that once activated, the CD4 cells in this transfer population are able to target and destroy $\beta 2m^{null}$ islet β cells. This suggests a role for substantial numbers of CD8 T cells in the effector phase of diabetes. Likewise, when NOD$\beta 2m^{null}$ mice are made transgenic for β2m on the rat insulin promoter (RIP), thus restoring MHC class I expression only on islet β cells, mice rapidly developed diabetes, following the transfer of diabetic spleen cells, at the same rate as wild-type NOD recipients (Kay et al., 1996). Interestingly, restoration of β-cell MHC class I, without restoring CD8 T cells, was sufficient to cause insulitis but not diabetes. This might be explained by previous findings that $\beta 2m^{null}$ mice possess small numbers of CD8 T cells

which are probably not functional in the absence of MHC class I expression (Lehmann-Grube et al., 1994; Zijlstra et al., 1992). In RIP-β2m/NODβ2m^{null} mice this small population may be able to initiate damage of β cells, yet remain too scarce to be involved in the final effector stage of diabetes.

C. T cell effector mechanisms

As previously discussed, CD4 and CD8 T cells play a role in both the initiation and the effector phase of β-cell destruction. It is not clear however, if the requirement for both CD4 and CD8 T cells in diabetes results from their synergistic or independent contributions. Furthermore, the mechanisms by which β cell destruction by CD4 and CD8 T cells occurs is not well established. It seems that CD8 T cell cytotoxicity results from the direct production of the effector molecule perforin, and granzymes. These molecules are also able to induce programmed cell death, or apoptosis. Apoptosis is also mediated by membrane-associated TNF-related molecules, such as Fas ligand, which interacts with Fas, its receptor, on target cells. Support for perforin-mediated β cell destruction comes from the LCMV-gp model. These mice are made perforin-deficient, and when LCMV-gp-specific T cells are activated following LCMV viral infection, they do not develop diabetes (Kagi et al., 1996), and perforin-deficient NOD mice have delayed onset of diabetes (Kagi et al., 1997). On the other hand, recent evidence indicates that apoptosis, as a result of Fas/Fas ligand interactions, in the NOD mouse may represent a dominant mode of islet β cell destruction by CD8 T cells (Chervonsky et al., 1997). Additionally, CD4 T cells have also been shown to destroy β cells. Since β cells do not express MHC class II molecules it is generally thought that CD4 T cell destruction is mediated indirectly, following their activation by APCs bearing β-cell-specific antigens. Alternatively, CD4 T cells could kill β cells by

inducing apoptosis. Kurrer et al (1997), using a TCR from the CD4 T cell clone BDC2.5, have shown that β cells die by apoptosis following interactions with BDC2.5 cells, and that death is triggered by a mechanism other than Fas/FasL, such as TNFα-TNFR interactions. Islet cells do not normally express Fas, but can be induced to up-regulate its levels by IL-1 and interferon-γ (IFN-γ) (Stassi et al., 1996; Yamada et al., 1996). Thus, cytokine production by CD4 and CD8 T cells may up-regulate Fas on islets, at which point Fas ligand-expressing CD8 T cells and CD4 cells would induce apoptosis (Wong and Janeway, 1997).

In earlier studies with NOD mice, investigators in our laboratory generated NOD mice that express the B7-1 co-stimulatory molecule, using the rat insulin promoter-1 (RIP), on pancreatic β cells. At the first backcross to NOD, mice homologous to the NOD MHC develop accelerated diabetes, with more than half the animals developing diabetes before 12 weeks of age. Under normal circumstances β cells do not express costimulatory molecules (Stephens and Kay, 1995). In this NOD-RIP-B7-1 model, it appears as though B7-1 expression allows β cells to be effective APC's. They become potent stimulators of CD8 T cells that are specific for β cell antigens presented by MHC class I molecules. Interestingly, both CD4 and CD8 T cells are required for the acceleration of diabetes in this model (Susan Wong et al., 1998).

It remains a possibility that T-cell activation in NOD mice does not require costimulation with B7 and could result from the interaction of CD8 T cells with B7-negative β cells. It is hypothesized that CD8 T cells are activated outside the pancreas in surrounding lymph tissue, where they recognize their antigen in the context of a costimulatory signal on the surface of APC's. These activated cells could then recognize their antigen within islets without the need of direct costimulation by B7 molecules (Wong and Janeway, 1997). There is increasing evidence that B lymphocytes may play a critical role in the

induction of diabetes, since their depletion causes resistance to both diabetes and insulitis (Noorchashm et al., 1997; Serreze et al., 1996). This effect likely relates to the ability of B cells to present antigen and costimulate T cells. On the other hand, it has recently been shown that dendritic cells, but not macrophages, efficiently present antigens derived from apoptotic cells which can then stimulate MHC class-I-restricted cytotoxic CD8 T cells (Albert et al., 1998). This may represent an exogenous pathway for CD8 T cell activation against target islet β cell antigens.

D. Diabetogenic CD4 and CD8 T cell lines and clones

With the advent of cloned T cell lines, it seemed that information on the relative contributions of CD4 and CD8 T cells to β cell destruction, as well as interactions between the two populations might be further delineated. However, the diabetogenicity of various lines and clones is apparently quite variable (Table 1). In addition, the requirement for both T cell subsets to cause diabetes is not as clear when T cell clones are used. In some reports, CD4 T cell clones can transfer disease but require the presence of CD8 T cells (Nagata et al., 1994; Pankewycz et al., 1991; Reich et al., 1989; Shimizu et al., 1993). On the other hand, several groups have isolated CD4 T cell clones (Daniel and Wegmann, 1996, Zekzer, 1997 #485; Peterson and Haskins, 1996) reactive to islets or known islet antigens, which can transfer diabetes in 4-7 weeks in the absence of other cells.

CD8 T cell clones are difficult to isolate, and consequently there are fewer reports of islet-specific CD8 T cell clones than there are CD4 clones. In addition, investigators have generally not observed disease transfer when CD8 clones are used alone, but only when they are cotransferred with CD4 lines or spleen cells (Nagata et al., 1994; Santamaria et al., 1995; Verdaguer et

al., 1996). Other researchers (Shimizu et al., 1993) isolated CD8 T cell clones from pancreatic islets of NOD mice which did not transfer disease even in the presence of CD4 T cells. However, investigators in our laboratory have recently generated an islet-specific CD8 T cell clone which has the capacity to cause diabetes rapidly (within 10 days) when transferred to irradiated NOD, young NOD-SCID, and CB-17-SCID mice, in the absence of CD4 cells (Wong et al., 1996).

These diabetogenic CD8 T cell clones were isolated from the pancreatic islets of young 7 week old NOD mice, which have insulitis but are not yet diabetic. They differ from other CD8 T cell clones in that they were grown on islets from B7-1-expressing (NOD X C57BL/6J-RIP B7-1)F1 hybrid mouse cells are highly cytotoxic, and express IFN-γ, TNF-α, TNF-β, and the effector molecule perforin. They rapidly destroy pancreatic islets in recipient NOD mice that do express B7-1 in their pancreases, indicating that B7-1 is not required for specific recognition or final effector function (Wong et al., 1996).

It has previously been demonstrated that T cells, capable of targeting and damaging islets, are present in the islets of young, pre-diabetic NOD mice (Rohane et al., 1995). It is important to note that these cells are highly cytotoxic, and express IFN-γ, TNF-α, TNF-β, and the effector molecule perforin. They rapidly destroy pancreatic islets in recipient NOD mice that do express B7-1 in their pancreases, indicating that B7-1 is not required for specific recognition or final effector function (Wong et al., 1996). It is important to note that the CD8 T cell clone isolated by Wong and colleagues (1996) was isolated from 7 week-old mice that had insulitis but would not be diabetic for several weeks. Therefore, these cells may represent a population of cells capable of inflicting significant damage to islets, and may be contributing to the initiation of the autoimmune process.

6. Autoantigens in type 1 Diabetes

Studies of NOD mouse from the neonatal period through to diabetes onset has led to view that the diabetogenic process is a step-wise development marked by two proposed 'checkpoints' (Andre et al., 1996). The first is seen at about 4-6 weeks of age, and is characterized by peri-insulitis. Following this, there is direct invasion of the islets by infiltrating cells, or intra-insulitis, which is dependent on recognition of β cell antigens (Wicker et al., 1992). A temporal analysis of β cell reactivity suggests that a few autoantigens are targeted early in insulitis (Kaufman et al., 1993; Tisch et al., 1993), and that intra-insulitis results in the recruitment of additional β-cell reactive T cells in the periphery. The onset of diabetes at 18-20 weeks represents the second checkpoint, and does not seem to be driven by insulitis per se (Katz et al., 1993). These events may depend on changes in regulatory and effector T cells, or possibly a cascade of antigen recognition, or both.

It has been about a decade since the identity of most known β cell autoantigens were determined. Despite this progress, many questions remained unanswered, particularly the intriguing question of whether a dominant, initiating antigen exists, and what is its nature. The roles of known β cell antigens are poorly understood, including whether they are in fact pathogenic. Particular β cell antigens in IDDM have been studied by first correlating autoantibody reactivity, and T cell reactivity, with disease progression in both human and NOD mice, and secondly looking at how diabetes in the NOD mouse can be modulated following antigen administration or transfer of antigen-specific T cell clones.

A. Glutamic acid decarboxylase

The above criteria have been used to characterize glutamic acid decarboxylase (GAD) as a critical β-cell autoantigen. Its two isoforms, GAD65 and GAD67, are the enzymes that catalyze the formation of the neurotransmitter, γ-aminobutyric acid. Autoantibodies to a 64 kilodalton (kd) protein were found to be present in diabetic patients in the 1980s (Baekkeskov et al., 1987). This antigen was subsequently identified as GAD, and is now used as a predictive marker in pre-diabetic patients (Baekkeskov et al., 1990; Hagopian et al., 1993). In the NOD mouse anti-GAD T cell responses appear very early on in insulitis, and prior to the appearance of T cell reactivities to other β cell antigens (including HSP60, and carboxypeptidase H) (Kaufman et al., 1993; Tisch et al., 1993). Thus anti-GAD activity may be connected to early events associated with insulitis. Furthermore, NOD mice are protected from diabetes when they are immunized early on with GAD (Elliott et al., 1994; Zechel et al., 1998) and seem to undergo a shift from a Th1-like response to a Th2-like GAD67-specific response. It has also been demonstrated that NOD mice exhibit spontaneous T cell reactivity to GAD (Kaufman et al., 1993; Quinn and Sercarz, 1996; Tisch et al., 1993). GAD-specific CD4 T cell clones have been generated (Schloot et al., 1996), and one such T cell clone has been shown to cause diabetes in NOD-scid mice (Zekzer et al., 1998). On the other hand, nasal administration of GAD was found to prevent diabetes, and down-regulate T-cell reactivity (Tian et al., 1996). Thus further work is needed in order to determine the relative contribution and precise role of anti-GAD reactivity in the disease process.

C. Insulin

Insulin is secreted, and along with C-peptide, comprises about 80% of the β-cell protein (Hutton, 1989). Thus its role as an autoantigen has been of great interest. It has been studied extensively and in fact appears to play an important role in diabetogenesis. Antibodies to insulin are found in about half of recent-onset diabetic subjects, and occur most frequently in children who have more aggressive β-cell destruction (Castano and Eisenbarth, 1990). The first report of insulin-reactive T cell clones was by Wegmann et al (Haskins and Wegmann, 1996), who isolated CD4 T cell lines and clones from 7 and 12 week old NOD mice, following their culture with islet cells as antigen. Of the CD4 T cell clones isolated from younger mice 14% of the cells were reactive to insulin. However, about half of the clones isolated from the older mice were insulin-reactive, the majority of which expressed a Th1-like phenotype, and one clone was found to transfer diabetes to NOD-*scid* mice. Most of these CD4 T cell clones respond only to the insulin B chain (Daniel et al., 1995), and NOD mice have shown diabetes resistance following the administration of this dominant epitope (Daniel and Wegmann, 1996; Muir et al., 1995). Unlike young NOD mice treated with GAD, histologic examination of mice receiving insulin continued to exhibit insulitis, which suggests that insulin reactivity correlates with a later phase of disease progression. More recently it was shown that the proinsulin II gene driven off the MHC class II promoter, to direct expression of proinsulin to MHC class II-bearing cells particularly in the thymus, almost completely prevents insulitis in transgenic NOD mice (French et al., 1997). Therefore, proinsulin also appears to play an important role in the pathogenesis of diabetes.

C. Other β-cell autoantigens

Several other β-cell proteins have been identified as T cell antigens. Autoantibodies and T cell reactivity directed towards heat shock protein (HSP) have been detected in NOD mice (Elias et al., 1990), and HSP60 reactive CD4 T cell clones were found to induce disease in NOD mice (Elias et al., 1991). However, it is not clear whether HSP is a target in human IDDM. Other target β-cell autoantigens identified in humans include carboxypeptidase H, islet-cell antigen (ICA) 69, and the tyrosine phosphatase IA-2/ICA512, found in the β-cell granule membrane. ICA512 is an important though less known ICA (Solimena et al., 1996), and anti-GAD and anti-ICA512 antibodies together are strongly predictive of diabetes in first degree relatives of diabetic patients (Bingley et al., 1997).

With the exception of insulin and proinsulin, few other molecules which are limited in their expression to β cells, have been well characterized (Hutton, 1989). However, evidence for an undefined membrane-associated antigen has been provided for CD4 islet-specific T cell clones (Bergman and Haskins, 1994). Subcellular locations of islet antigens were analyzed, and it was found that a fraction which was highly enriched in β-cell granules was antigenic for every T cell clone tested. They subsequently localized that activity to the β-cell granule membrane.

Many important questions regarding the nature and role of β-cell autoantigens remain to be answered. At present it seems that rather than a single, organ specific antigen, many antigens play a role in human IDDM and NOD mice, and many more unidentified β-cell autoantigens likely exist. One important unanswered question is whether there exists a primary β-cell antigen that dominates the induction of diabetes, and if it does exist, will its identification have therapeutic implications? It seems unlikely that the

various autoantigens identified thus far could be initiating the same primary diabetogenic response. Rather, it is likely that a particular type of antigen is responsible for disease initiation, leading to local inflammation, and recruitment to the pancreas of islet-reactive T cells with more diverse specificities (Haskins and Wegmann, 1996).

To date, research has focused on the generation of CD4 T cell clones that are reactive to previously identified β cell autoantigens. As discussed above, it now appears that CD8 T cells may be critical initiators of diabetes. Thus, it is likely that the antigenic targets of CD8 T cell also contribute to IDDM induction. However, no CD8 T cell antigen has yet been identified in human or NOD mouse IDDM. Elucidating the nature of CD8 T cell autoantigens is important for further understanding IDDM in NOD mice and humans, developing treatment modalities, and may lead the discovery of a dominant primary diabetogenic antigen.

Table 1. T cell clones from the NOD mouse (table adapted from Bergman and Haskins, 1997).

Origin of T cells	CD4/CD8	Antigen specificity	*in vivo* activity	Investigators
Spleen and lymph nodes of diabetic females	CD4	Islet cells, β granule membrane	Insulitis & diabetes	Haskins et al, 1988, 1989. Bergman & Haskins 1994
Islet infiltrates of diabetic females	CD4 & CD8	Islets cells	Insulitis	Reich et al, 1989
Islet infiltrates of diabetic females	CD4	Unknown	Protection	Reich et al, 1989
Islet infiltrates of 20-week old pre-diabetic females	CD8	Islet cells	Not determined	Nagata et al, 1989
Three month old female	CD4	HSP-65	Insulitis	Elias et al, 1990
Islet infiltrates of 8-week females (ND)[a]	CD4	Islet cells	Protection	Pankewycz et al, 1991
Islet infiltrates of 8-week females (ND)	CD8	Unknown	Insulitis	Pankewycz et al, 1991
Islet infiltrates of 7-10 week old mice (ND)	CD4	Islet cells	Insulitis	Nakano et al, 1991
Islet infiltrates of <10 & > 10 week old females (ND)	CD4 & CD8	Islet cells	Diabetes (CD8 co-transferred with CD4)	Nagata and Yoon, 1992. Nagata et al, 1994
Islet infiltrates from irradiated male recipient	CD4 & CD8	Islet cells	Diabetes (CD4 clones only	Shimizu et al, 1993
Islet infiltrates of 10-12 week old females (ND)	CD4	Unknown	Not determined	Maugendre et al, 1993
Islet infiltrates of 4-week old females (ND)	Not determined	Unknown	Protection	Chosick & Harrison, 1993
Infiltrated islet allographs	CD4	Islet cells	Insulitis and diabetes	Wegmann et al, 1993
Islet infiltrates of 4 & 7 week old females (ND)	CD4	Islet cells, insulin	Insulitis and Diabetes	Wegmann et al, 1994
Spleen & lymph nodes of 30-40 day old females (ND)	CD4	Insulinoma extracts	Insulitis	Gelber et al, 1994
Lymph nodes of 6-week old or diabetic females	CD4	GAD-67	Not determined	Elliot et al, 1994
Lymph nodes of diabetic females	CD4	Islet cells	Insulitis and Diabetes	Healey et al, 1995
Spleen cells of 4-week nondiabetic females	CD4	Islet cells	Protection	Akhtar et al, 1995
Islet infiltrates of 7 week old females (ND)	CD8	Islet cells	Diabetes	Wong et al, 1996
Spleens of 6 week old diabetic mice, previously immunized with GAD	CD4	Islet cells	Insulitis and Diabetes	Zeckzer et al, 1998

[a] ND; Non-Diabetic

7. Measuring T Cell Activation

Overview

T cell activation takes place when a TCR of a circulating lymphocyte discovers and binds to its peptide/MHC ligand. Many biochemical events, both cytoplasmic and nuclear take place during T cell activation (Cantrell, 1996). Early cytoplasmic events such as protein tyrosine kinase cascades, cleavage of inositol phosphate by phospholipase C, and the mobilization of intracellular calcium stores, lead to the expression of numerous genes required for T cell proliferation and effector functions. Transcription of the interleukin II (IL-2) gene occurs early on in T cell activation, and in fact is thought to be the first committed step of the activation pathway (Crabtree, 1989). Following antigen recognition by the TCR, a transcription factor called nuclear factor of activation in T cells (NFAT) binds to the promoter region of the IL-2 gene. The NFAT binding site (Figure 2) is believed to be the most important response element for TCR-mediated transcription of the IL-2 gene (Shaw et al., 1988).

A. Bulk T cell activation assays

The detection and quantitation of T cell activity, and thus any experimental strategy aimed to identify the peptides recognized by immunologically important TCRs, has been hampered by the fact that most T-cell activation assays have low sensitivities. Measuring cytotoxic T cell activity *in vitro* includes such techniques as ^{3}H-thymidine proliferation assays, or in the case of cytotoxic T cells, quantifying killing of target cells via ^{51}Cr-release assays. Assays which quantitate lymphokine production, including the IL-2, TNF, and INF-γ assays, are commonly used. More recently, the development of calcium sensitive dyes has enabled the

visualization of calcium mobilization during T cell activation (Poenie et al., 1987). However, calcium fluxes do not always correlate with T cell activation, and so calcium levels are not as reliable measure of T cell activity as are the IL-2, proliferation, and cytoxicity assays (Sussman et al., 1988).

These assays provide a rapid and specific measurement of cytotoxic T cell activity, yet they have a significant drawback. With the exception of calcium sensitive dyes, these T cell activation assays detect compounds released by T cells or APCs into the supernatent, and thus measure the average activation state of all T cells in the assay. They provide no information regarding the activation state of individual T cells, and cannot be used to detect activated T cells when they form only a small proportion the total T cell population being assayed. This limits the sensitivity of bulk T cell activation assays for detecting peptide/MHC-expressing APCs .

B. NFAT lacZ reporter construct and the lacZ assay

In this thesis we have taken advantage of a recently introduced T cell activation assay (Karttunen and Shastri, 1991), which utilizes a reporter construct consisting of the bacterial *lacZ* gene under control of the NFAT element of the human IL-2 enhancer (Figure 2). The *lacZ* gene encode β-galactosidase, an enzyme that acts on chromogenic substrates such as 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). *LacZ* activity is induced in T cells by the interaction with peptide/MHC ligand, and parallels IL-2 expression. Once activated, T cells transfected with the *lacZ* reporter express uniformly high levels of β-gal and turn blue in the presence of X-gal. This enables the detection of individual activated cells, even when they are a small fraction of the total T cell population. This additionally allows for the detection of rare APCs which express the activated T cell's target antigen.

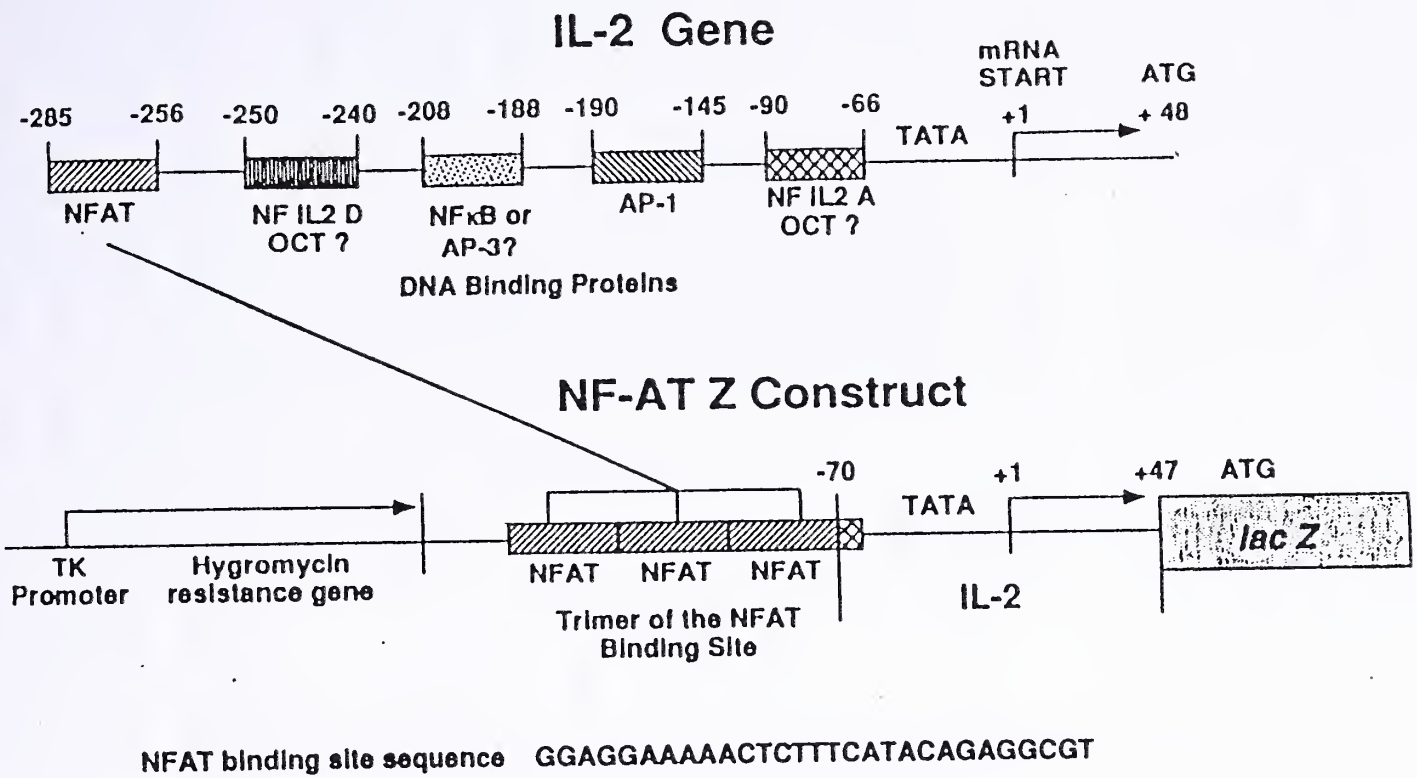


Figure 2. Diagrammatic structure of the NFAT-*lacZ* reporter construct, using the enhancer/promoter of the human IL-2 gene. (Figure from Fiering et al., (1990) Genes and Development 4:1823-1834.)

8. Strategies for identifying T cell antigens

Overview

There are two general strategies for the identification of the peptide-MHC ligands recognized by T cells: biochemical purification of MHC-bound processed peptides, and expression cloning of the antigenic genes. Both approaches require that the MHC-peptide ligand be expressed on the surface of APCs, and be detectable by a T cell activation assay.

A. Biochemical purification of antigens

T cell antigens can theoretically be identified by directly purifying naturally processed and presented MHC-bound peptides from target APCs. However, biochemical purification is difficult not only because MHC molecules display thousands of different peptides on the cell surface (Falk et al., 1991; Hunt et al., 1992), but also because only a few copies of individual antigenic peptides may be presented per cell (Cox et al., 1994; Kageyama et al., 1995). In addition, it is noteworthy that the peptide purification strategy requires sophisticated instrumentation, (such as a high pressure liquid chromatography (HPLC) system and a mass spectrophotometer), not to mention experimental and analytic skills that are not available in many laboratories.

Nevertheless, several groups have overcome these difficulties and have identified the antigenic ligands of some T cells using HPLC. Eisen and co-workers identified the peptide component of the 2C ligand alloantigen (graft rejection antigen) recognized by C57BL/6 T cells on BALB/c mouse tissue (Udaka et al., 1992; Udaka et al., 1993). A human alloantigen associated with HLA-A2.1 class I molecule (Henderson et al, 1993), as well as a peptide recognized by melanoma-specific human cytotoxic T cell lines (Cox et al.,

1994), were similarly isolated by Hunt and colleagues. However, unlike alloantigens and tumor cell proteins which can be relatively abundant, most immunologically important peptides, including those involved in autoimmunity, are present at very low frequencies on cell surfaces, and have been resistant to isolation by this approach.

The identification of autoantigens in type 1 diabetes using biochemical analysis is particularly difficult because the isolation of mouse islets is a very expensive and labor intensive endeavor. The typical yield from a NOD mouse pancreas is in the order of 10^5 islet cells. Thus the biochemical purification of islet cell proteins, which requires in the order of 10^{10-12} islet cells (Shastri, 1996), is not feasible without a tumor cell source. Unfortunately, tumor cell lines are known to undergo a variety of changes when kept in culture, and tend to quickly lose their antigenicity. Researchers who have attempted to identify T cell antigens using biochemical techniques have thus needed to use mouse strains that produce β-cell tumors (insulinomas) (Bergman and Haskins, 1997). However, this antigenic source may similarly not be representative of the proteins naturally existing in NOD mouse islets.

B. Expression cloning of T cell antigens

In contrast to biochemical purification, expression cloning strategies are not dependent upon the total amount of obtainable peptide, but upon the construction of representative cDNA libraries, the efficient expression of candidate antigen genes, and the generation of appropriate peptide-MHC ligands on the APCs. The much smaller amounts of tissue required to make a cDNA library, as compared to the amount needed to purify adequate quantities of peptides, is especially useful when antigen-expressing tissue, as

with the pancreatic islets of NOD mice, is particularly difficult to obtain. Not only does this approach require less starting materials, the DNA manipulations required for expression cloning are now routinely available in most laboratories (Shastri, 1996). For these reasons we have chosen to use expression cloning, rather than peptide purification as a means to identify the islet cell antigen of our diabetogenic CD8 T cell clones.

C. Expression cloning of a CD8 T cell antigen

The isolation of CD8 T cells and identification of their autoantigenic peptides is a difficult task. Thus very few CD8 clones have been generated, and their target antigens have remained elusive (Nagata et al., 1994; Santamaria et al., 1995; Shimizu et al., 1993). The primary difficulty with identifying T cell antigens, which relies on the detection of ligand-induced T cell activation, arises from the fact that neither the TCR nor its peptide/MHC ligand exist functionally in a soluble state. Since both the receptor and its ligand are membrane-bound, screening for a T cell antigen must rely on cellular assays, the majority of which are inherently insensitive, and cannot detect small populations of activated T cells. The work of Jaana Karttunen in developing the *LacZ* assay further suggested an expression cloning strategy that exploits the highly sensitive *lacZ* inducible T cell hybrids (Karttunen et al., 1992; Sanderson and Shastri, 1994). This cloning strategy, originally developed by Aruffo and Seed, is based on the transient expression of candidate cDNAs in COS cells (Aruffo and Seed, 1987a, 1987b).

COS cells are transformed cell lines generated by the transfection the CV-1 simian fibroblast line with an origin defective mutant of the SV40 virus (Gluzman, 1981). These cell lines stably express the SV40 large T antigen. As such they are capable of replicating circular DNAs containing the SV40 origin

of replication, and attain very high levels of protein expression. Using the *lacZ* assay, it has been shown that COS-7 cells can function as APCs for the detection of rare antigens found in the primary screen of expression libraries; antigen-expressing COS-7 cells can be detected at frequencies of $1:10^3$ to $1:10^4$ (Karttunen et al., 1992). Since T cells recognize peptide in the context of self-MHC molecules, COS cells must first be stably transfected with the relevant MHC molecule.

We are applying this expression cloning system as a means to identify the nature of the antigen recognized by the CD8 cell lines in young NOD mice (Wong et al., 1996). The system consists of *lacZ* inducible T-cell hybrids specific for the unknown antigen, and a COS cell line expressing the relevant MHC. In this case the H-2K^d restriction element recognized by the CD8 T cell clones has been stably transfected into COS-7 cells (K^d-COS). These APCs, in the presence of *lacZ* inducible hybrids, were used to screen a cDNA library that was generated from normal islet β cells which express the autoantigen (Figure 3).

Statement of purpose

The definitive identification of the autoantigens involved in IDDM is an important initial step in sorting out the series of events leading to the acquisition of the disease. Despite significant efforts to identify the specific antigenic peptide recognized by an individual TCR, this has remained a difficult undertaking. The primary reason for this difficulty lies in the inherent lack of sensitivity of the bulk T-cell activation assays used to detect peptide/MHC-expressing APCs. These assays only measure the average state of activation within a given T cell population, and thus cannot detect activated T cells when they constitute only a small percentage of the total T

cells being assayed. The research presented here is the screening of an expression cDNA library made from normal NOD mouse islets which express the autoantigen of cloned CD8 T cells, obtained at an early stage of disease in NOD mice. This requires the ability to detect ligand-bearing APCs when they are present at very low frequencies. In recent years a method for detecting individual ligand-activated T cells that are stimulated by correspondingly rare MHC class I restricted APCs has been developed. This method has been used here as an expression cloning strategy for identifying the target antigen(s) of the diabetogenic G9 CD8 T cell clone.

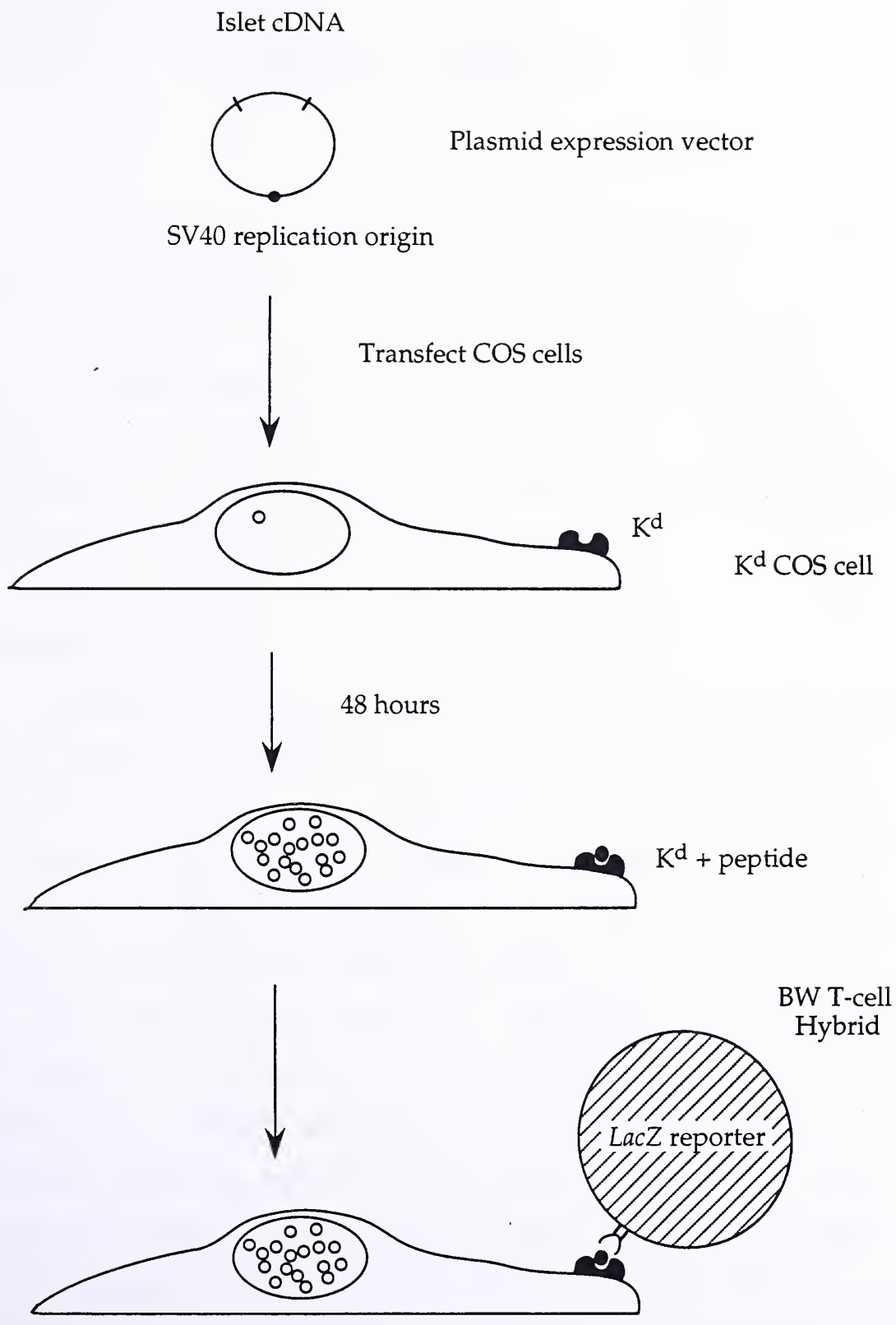


Figure 3. Schematic illustration for processing and presentation of transiently expressed proteins by K^d-COS cells to the BW/G9 T-cell hybrid.

Materials and Methods

1. Cell culture

COS-7 cells (ATCC) are maintained on Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, penicillin (200 units/ml), streptomycin (200 ug/ml), and streptomycin (200 μg/ml). K^d-COS cells were maintained on DMEM with supplements, plus 1 μg/ml G418 (Geneticin). T cell hybrids are maintained on Click's with 10% fetal bovine serum, plus antibiotics. All cells are kept at 37°C in a 9% CO_2/91% air atmosphere.

2. Cell lines.

As indicator T cells, this system uses α-β-BW5147 cells, transfected with the NFAT-*lacZ* construct by electroporation (obtained from Dr. N. Shastri). These cells were transfected with a CD8α retrovirus and have been fused to the diabetogenic CD8 T cell clone G9 (Wong et al, 1996), generating hybrid BW/G9 cells which express the Vβ6 TCR as well as CD8 and CD3, together with the *lacZ* gene driven by the NFAT promoter.

COS-7 cells were obtained from ATCC. The cells were stably transfected with a genomic clone of the murine H-2K^d molecule, kindly provided by J. Karttunen (Yale University School of Medicine). Using the Superfect transfection reagent (see below), COS-7 cells were transfected with K^d plasmid along with a co-selection marker pMC11neo-polyA+ plasmid which confers neomycin resistance. Transfectants were selected in 1 μg/ml of G418 (Geneticin), and K^d expressing COS cells were isolated by fluorescence-activated cell sorting (FACS). The most highly expressing K^d-COS cells were selected and subsequently cultured.

3. Transfection protocols

COS-7 cells were transfected using Superfect (Quiagen) which represents a new class of polycationic transfection reagent, and offers high efficiencies and reproducibility, with minimal toxic effect. Stable or permanent transfection was carried out using adherent COS cells that were 50% confluent in 50 ml tissue culture flasks. 10 µg of DNA, complexed with Superfect, was transferred to the COS cells. The cells and complexes were incubated for 2 to 3 hours at 37°C in 5% CO_2 atmosphere. They were then washed 4 times with 10 ml of PBS, covered with fresh growth medium, and incubated for 24 hours. Transfectants were subsequently selected with 0.5 µg/ml G418 for 24 hours, followed by 1 µg/ml G418.

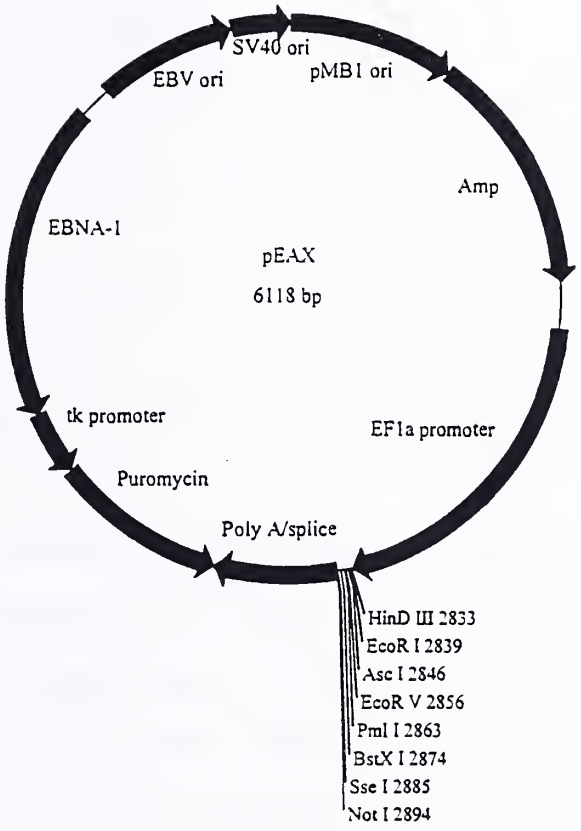
The same protocol was used for transient transfections, though it was modified for use in flat-bottomed 96-well plates. Approximately 1 x 10^4 K^d-COS cells per well were plated the day prior to transfection. For each plate, 35 µg of DNA and 150 µl of Superfect reagent was allowed to complex in 750 µl of antibiotic and serum free DMEM. Following incubation, this mixture was brought to a total volume of 6 ml with appropriate medium, and 50 µl of the mixture was then dispensed into each of the 96 wells of washed K^d-COS cells. The cells and DNA-Superfect complexes were incubated for 2-3 hours at 37°C in 5% CO_2 atmosphere, washed 3 to 4 times with 150 µl of sterile PBS and then covered with 100 µl of growth medium per well. Plates were incubated for 48 hours before being assayed for *lacZ* activity.

4. FACS staining

K^d-COS cells were stained for FACS analysis using the monoclonal antibody HB159 (anti-H-$2K^d$). About 10^5 transfected cells, and untransfected control COS-7 cells, were each incubated in the dark for 30 minutes at 4°C with the primary HB159 antibody (diluted 1:200). Cells were then spun at 2000 rpm for 5 minutes, washed once with staining buffer, and respun. The secondary antibody, anti-mouse IgG (FC specific) FITC (diluted 1:500), was then added to the cells, which were again incubated for 30 minutes in the cold and dark. Finally, the stained cells were spun down and fixed with 1% paraformaldehyde.

5. cDNA library construction

The cDNA library of NOD mouse islet β cells, containing the mRNA of the unknown peptide, was made using 12 μg of NOD islet mRNA. The mRNA was extracted from the islets of 200 young male NOD mice (work performed by M. Altieri). The islets were isolated by collagenase digestion and purified on Percoll density gradients. Total RNA was extracted using Trizol reagent (Gibco) and mRNA was further purified using a poly A column (carried out by S. Wong). The cDNA library is an unamplified library, and consists of > 3×10^7 primary transformants, which are DH10B bacterial cells. The library was provided in sixty, 1 ml pools each containing > 5×10^5 clones (synthesized by Edge Biosystems AGTC, Maryland). The library was constructed in the expression vector pEAX, which contains the SV40 origin of replication for plasmid expression in SV40 large T antigen-expressing COS cells (Figure 4). The average cDNA insert size is 1.31 kilobases, and the insert cutoff size is 700 base pairs.



Sequence flanking cloning site of pEAX

```
2701  CTGAAGTTAG GCCAGCTTGG CACTTGATGT AATTCTCCTT GGAATTTGCC

2751  CTTTTTGAGT TTGGATCTTG GTTCATTCTC AAGCCTCAGA CAGTGGTTCA

2801  AAGTTTTTTT CTTCCATTTC AGGTGTCGTG AA AAGCTT GA  ATTC GGCGCG
                                        Hind III  EcoR I    Asc I

2851  CCA GATATC A CACGTG CCAA GGGGCTGG{cDNA 5'- 3'}CA CCTGGCCTGC
        EcoR V    PmI I     Bst XI                          Sse I

      AGGCGGCCGC
        Not I

2900  AGGTAAGCCA GCCCAGGCCT CGCCCTCCAG CTCAAGGCGG GACAGGTGCC

2951  CTAGAGTAGC CTGCATCCAG GGACAGGCCC CAGCCGGGTG CTGACACGTC

3000  CACCTCCATC TCTTCCTCAG GTCTGCCCGG GTGGCATCCC TGTGACCCCT

3051  CCCCAGTGCC TCTCCTGGCC CTGGAAGTTG CCACTCCAGT GC
```

Figure 4. pEAX vector of the islet cDNA library, and the sequence cloning site of pEAX.

6. COS cell transient expression assay

Individual cultures of H-2K^d-COS cells, transiently transfected with pools of cDNA, were set up in 96-well plates. The cells were incubated for 48 hours to allow expression of the introduced genes. After 24 hours, about ten NOD mouse islets were added to each of ten wells of the negative control plate (see below). Another 24 hours later, 2 X 10^4 G9 T cell hybrids per well were added to the transfected COS cells to give a total volume of 0.2 ml per well.

The T cell hybrids were co-cultured overnight with cDNA-expressing K^d-COS cell, and then fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma). Plates were washed once with 0.1 ml of PBS per well, spun for 1 minute at 2000 rpm, and then fixed for 5 minutes on ice with 0.1 ml of ice-cold 2% formaldehyde/0.2% gluteraldehyde in PBS per well. After another wash with PBS and spinning, the cells were overlaid with a solution of 0.5 mg/ml of X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$. After an overnight incubation at 37°C, the plates were then examined microscopically for blue (*lacZ* - expressing) hybrid T cells.

7. cDNA Library Screening.

Having generated both the expression library and indicator T cells, large pools of cDNA's were screened for autoantigen-expressing APCs. This method has been shown to detect ligand-expressing APC's at minimum frequencies of 1 in 10^3 (Karttunen et al., 1992). Large pools containing 5 x 10^5 cDNAs were initially screened. Since NFAT-mediated transcriptional activity is an early event in T cell activation, the *lacZ* response is detectable within 6 hours.

Controls were included in each set of transfection assays. Negative controls consisted H-2K^d COS cells transfected with P022, the DNA sequence of ovalbumin. T cell hybrids, incubated with irradiated NOD mouse islets and control-transfected COS cells for 24 hours, served as positive controls. When wells contained clusters of blue cells, or a significantly greater number of blue cells in a well than the negative control plate, they were treated as potentially positive pools; that is, the cluster of blue hybrids had been activated by a peptide presented by the transfected COS cells.

Each potentially positive pool was further tested for the presence of an activating message using the process of limited dilution (Figure 5). After several subdivisions a pool with approximately 200 cDNAs was obtained. From these small pools, the plasmid cDNA from single, hand-picked bacterial colonies were purified and transfected into single wells of K^d-COS cells. When individual cDNA plasmids expression in K^d-COS cells led to the activation of significant numbers of BW/G9, the cDNAs were identified as candidate antigens. In order to define their nature they were sequenced, and compared to known gene and amino acid sequences using the NCBI database and Swiss protein database, respectively. Subsequently, the minimal peptide determinants within these sequences were identified by comparing them to K^d-specific motifs (Falk et al., 1991), which are defined by a tyrosine residue at position 2, and leucine or isoleucine at position 9 of the peptide. These sequences are then used to synthesize the minimal peptides. The antigenicity of these peptides was subsequently evaluated using proliferation and ^{51}Cr cytotoxic assays with G9 T cell clones, as well as in *LacZ* assays with K^d-COS cells and BW/G9 hybrids.

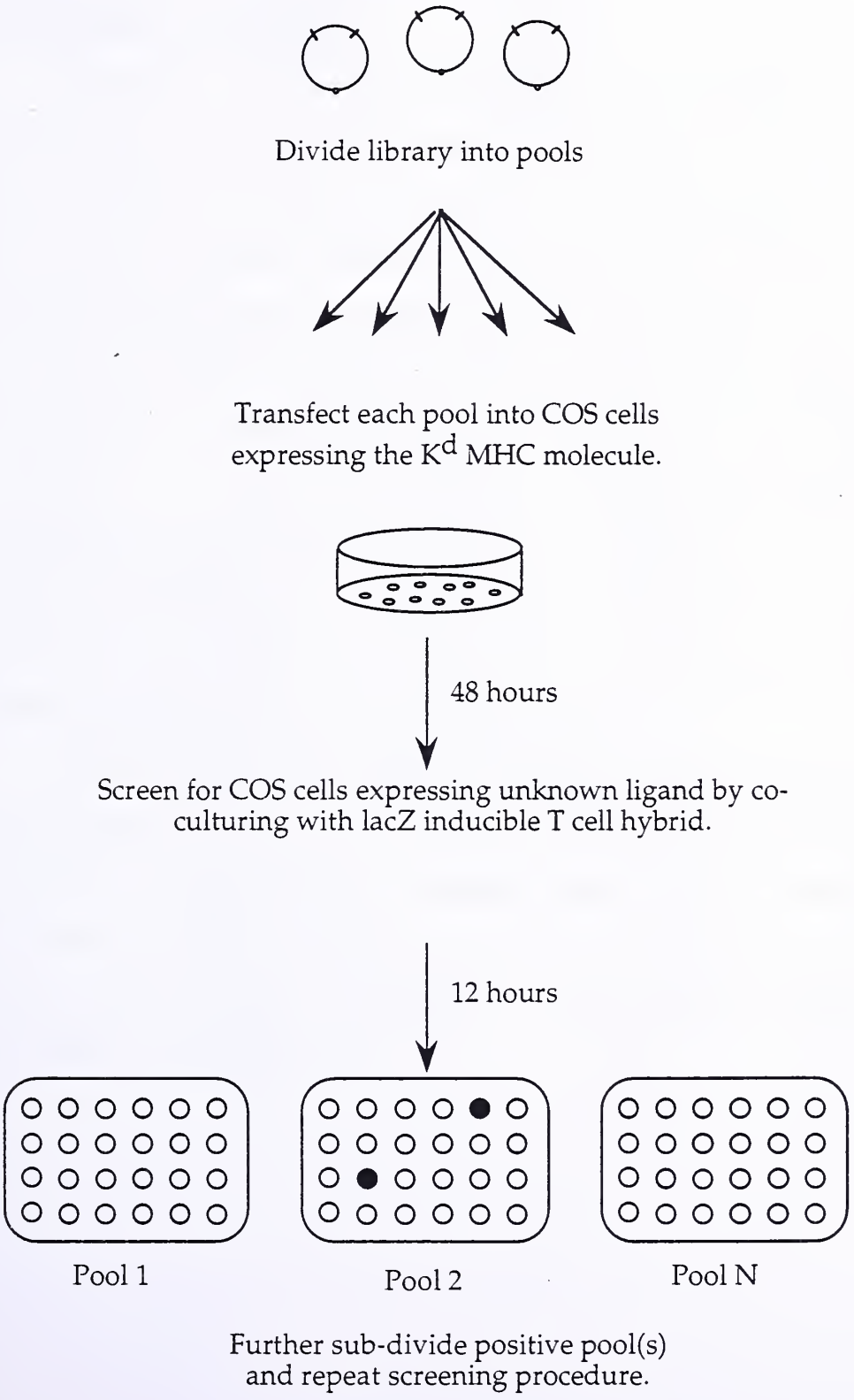


Figure 5. Schematic illustration summarizing the strategy for expression cloning of T-cell antigens.

8. cDNA pool subdivision

Pool subdivisions were accomplished by transforming XL-1 blue electroporation-competent cells (Stratagene, California). The original 60 pools each contained 5 x 10^5 cDNAs in bacteria. Xl-1 blue cells were transformed by electroporation with DNA such that ten fold fewer (ie. 5 x 10^4) colony forming units (CFUs) each receiving a different cDNA plasmid, grew on each of ten 15 cm x 15 cm Luria-Bertani (LB) agar containing 40 µg/ml ampicillin plates. The XL-1 blue cells were thawed on ice, of which 40 µl was mixed with diluted DNA. Cells were transferred to a chilled 0.4 cm-gap electroporation cuvette, and pulsed once with 2500 volts on a Bio-Rad electroporator, set at a resistance of 200 Ohms and capacitance of 25 µF. 960 µl of LB was immediately added to the cells, which were then allowed to recover for 1 hour at 37°C before being plated (96 µl/plate) as described above.

Bacterial colonies were scraped off the plates, resuspended, and plasmid DNAs purified using the TENS mini-prep method for use in subsequent transfections of H-2K^d COS cells. Since the pools were ten-fold smaller, they were screened with the *lacZ* assay in only a third of a 96-well plate. This process of limited dilution was repeated each time a positive signal was identified in a subdivided pool.

Results

1. Stable transfection of COS cells with the H-2K^d MHC class I molecule yields K^d expressing APCs.

In order to screen the islet cell cDNA expression library, we took advantage of the well-developed transient expression system present in COS-7 cells (Aruffo and Seed, 1987; Aruffo and Seed, 1987) as described by Karttunen and Shastri (1991). COS cells are transformed cell lines generated by transfecting the CV-1 simian fibroblast line with an origin-defective mutant of the simian virus 40 (SV40). These cell lines express the SV40 large tumor antigen and support the replication of circular DNA containing the SV40 origin.

In order to generate a COS cell line that can present peptides/K^d ligands to the K^d class I MHC-restricted T cell hybrids, COS cells were stably transfected with the K^d molecule. The K^d-expressing COS cells (K^d-COS) were analyzed by flow cytometry and found to express K^d at high levels (Figure 6). Though the ability of K^d-COS cells to present endogenous proteins to BW/G9 hybrid cells was not tested, it has previously been determined that K^b-COS cells process and present transiently expressed ovalbumin (OVA) to an OVA/K^b restricted T cell clone (Karttunen et al., 1992). This demonstrates that cDNA plasmids are expressed and presented to T cells by COS-7 cells, and that COS-7 cells can function as efficient APCs for MHC class I-restricted NOD mouse T cells (diagrammed in Figure 3).

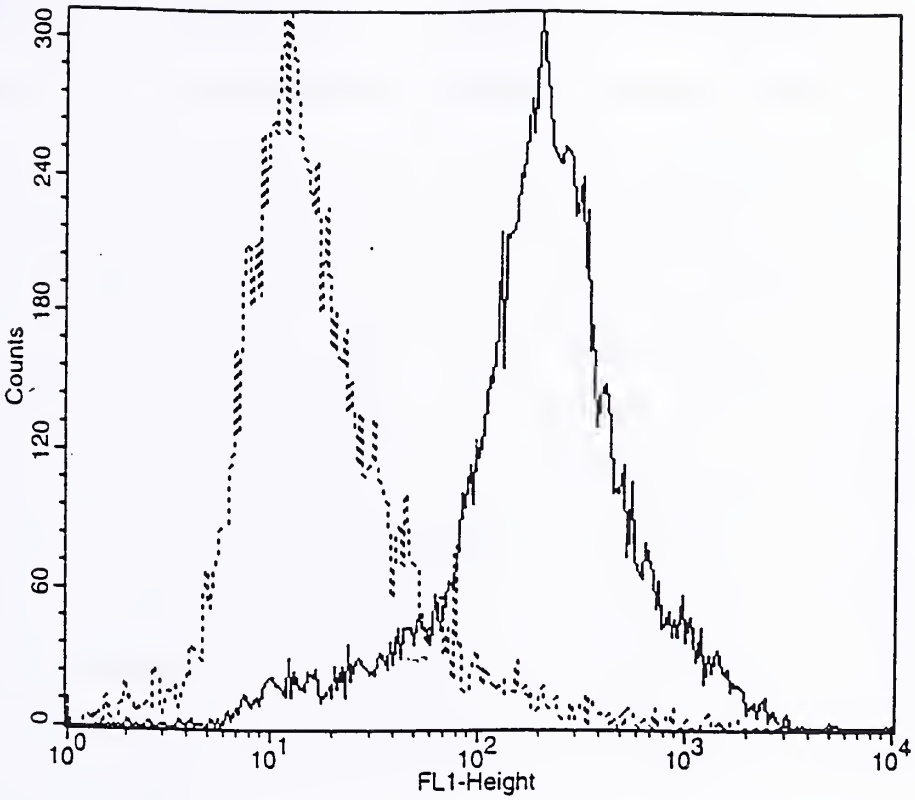


Figure 6. COS-7 cells transfected with MHC class I molecule H-2K^d, express K^d on their surface, as shown by staining with anti-K^d antibody HB519 (——), as compared to untransfected COS cells stained with anti-K^d (·····).

2. Microscopic detection of *lacZ* -inducible T-cell hybrids in the presence of NOD mouse islets and the chromogenic substrate X-gal.

The analysis of K^d-COS cells transfected with islet cDNA was performed using the *lacZ*-expressing T cell hybrids. Indicator T cells were visualized by staining with the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). X-gal is cleaved by β-galactosidase to yield 5,5'-dibromo-4,4'-dichloroindigo, which stains the *lacZ* -expressing T cells dark blue. It has previously been demonstrated by Karttunen et al (1992) that X-gal staining, used as a semi-quantitative or qualitative assay, is very sensitive for detecting activated, *lacZ*-expressing T cells, and therefore their corresponding ligand-expressing APCs. In those studies, ligand expressing K^b-COS cells were detected at a frequency of $1:10^3$, when assayed in triplicates (Karttunen et al., 1992).

The photomicrograph of X-gal-stained control samples, shown in figure 7, illustrate how activated *lacZ*-expressing BW/G9 T cell hybrids are visualized as darkly staining blue cells, and are easily distinguishable from background, yellow APCs and non-activated T cell hybrids. Figure 7 illustrates that BW/G9 T cell hybrids are islet specific; they turn blue in the presence of NOD mouse islets, which serve as a positive control for the *LacZ*/COS cell assay. As a negative control, BW/G9 cells are co-cultured with ovalbumin-expressing K^d-COS cells transfected with the OVA (P022) gene, and rarely turn blue (Table 2). The negative control illustrates that BW/G9 cells do turn blue in the absence of the antigen, and this represents background *lacZ* activity that is significantly less than that seen with clones cultured in the presence of antigenic islets.

It was observed that with time BW/G9 T cell hybrids gradually lost their ability to turn blue in the presence of islets. Hybrid T cells tend to

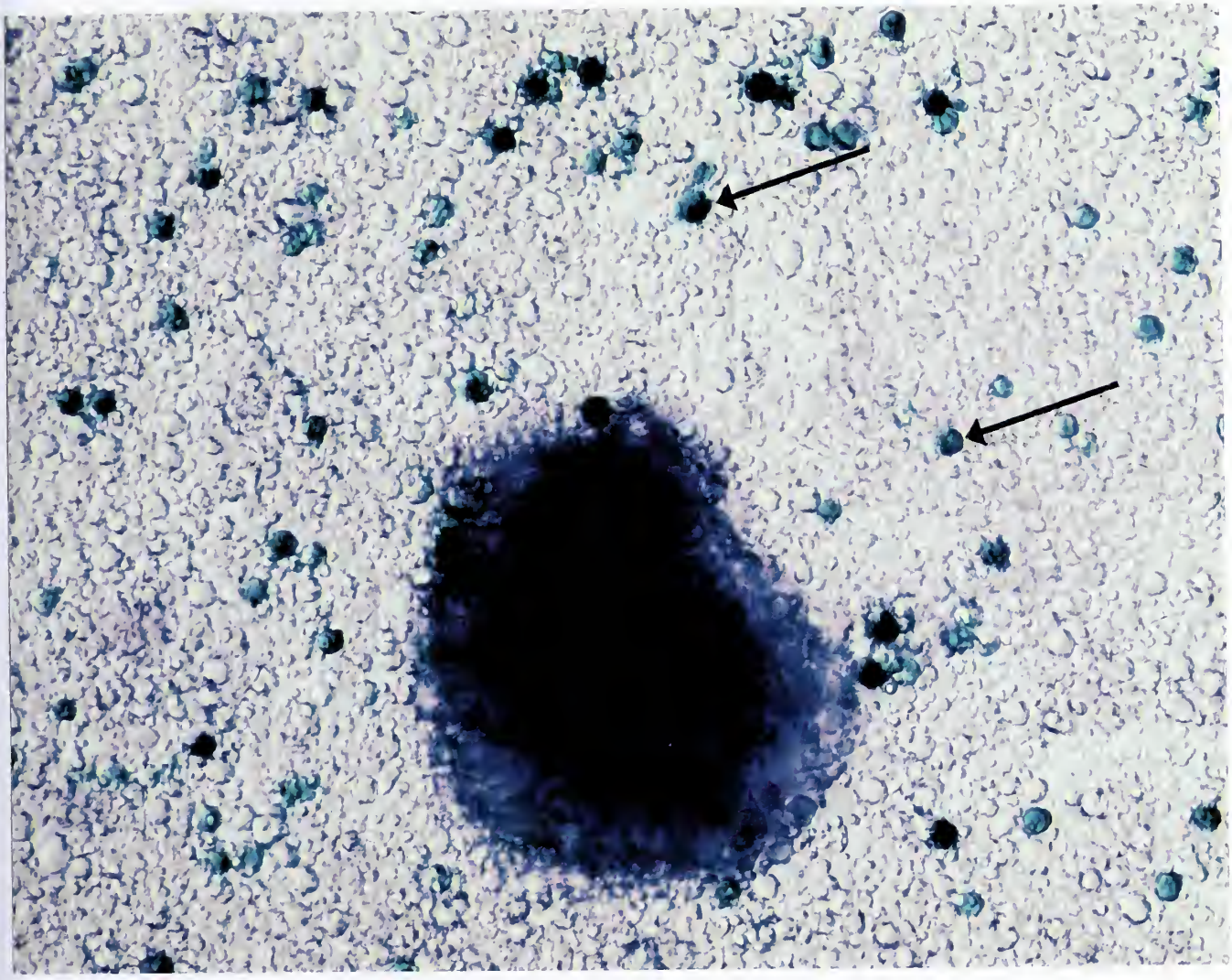


Figure 7. *LacZ*-inducible T cell hybrids turn blue in the presence of islets and the chromogenic substrate X-gal. Arrows indicate activated (blue) cells surrounding a NOD mouse islet.

proliferate extremely rapidly, and after 4 or 5 weeks in culture have passed through many cycles of cell division. It is unclear why the *lacZ*-expression of T cell hybrids became less inducible with time, though it may be related to the observation that T cell hybrids are inherently unstable. They tend to lose their TCRs, which would result in decreased reactivity to their ligands after several generations in *vitro*. Thus, T cell hybrids were discarded after about a month of culture, and fresh batches of frozen T cell hybrids were thawed and used in future assays. In addition, the background *lacZ* activity seen in the negative control assays was highest with newer T cell hybrids. The frequency of background X-gal stained T cells gradually decreased with time, thus paralleling the decrease in T cell activity seen in the positive controls.

3. A pool of islet cDNA induces *lacZ* -expression in BW/G9 T cell hybrids.

Using the *lacZ*/COS cell assay all 60 pools each containing about 5×10^5 unique cDNA were screened. One pool of the islet cDNA library, when transfected into K^d-COS cells, appeared to induce increased *lacZ* activity in the islet antigen-specific T cell hybrids; about 20 blue cells per well, versus 2-3 blue cells per negative control well (Table 2). The antigenicity of the cDNA pool was suggested by the fact that there were increased numbers of blue, X-gal stained cells as compared to the negative control. However, although all of the T cells turned blue with equal intensity, the number of *lacZ*-expressing T cells in this pool did not approach that seen in the positive control. When this pool was subdivided into ten, smaller pools each containing 5×10^4 transformants, and re-screened, similar numbers of activated T cells (about 5-10 fold more than the negative control) (Table 2) were again visualized.

Table 2. Representative numbers of *lacZ* expressing (blue) indicator T cells per well of potentially positive cDNA pools, and corresponding numbers in control (P022 expressing) wells. Counts are either numbers of clustered blue cells or the average number of blue cells/well in 5 wells.

cDNA pool #	# blue cells/well	# blue cells/control well
6	10 (cluster)	1-2
14	6 (cluster)	1-3
24	12 (cluster)	2-3
24 (s.d.)[a]	40	3-4
24 (s.d.)	36	3-4

[a]s.d.; subdivided

Thus, we continued this process of limited dilution, each time subdividing the pool which had yielded the most numbers of activated BW/G9 cells.

4. Establishing the antigenicity of candidate cDNAs

When a pool size of about 2 x 10^2 colonies was reached, individual cDNAs were transfected into wells of K^d-COS cells, and screened. Several such candidate cDNAs were associated with a 10-fold increase in *lacZ* activity above background, and were selected as candidate antigenic cDNAs, sequenced, and compared to known gene and amino acid sequences using the NCBI database and Swiss protein database, respectively. Three of these cDNAs were identified as the mRNAs coding for amylase, pre-proglucagon, and pre-proinsulin. However, when the corresponding peptides of known K^d motifs within these genes were tested for their antigenicity on K^d-COS cells and NOD spleen cells using proliferation assays with G9 clones, as well as *lacZ* assays with BW/G9 hybrids, no T cell activation was detected.

Furthermore, they candidate genes were re-tested by transfecting the cDNA into K^d-COS cells and again no significant β-gal activity was detected in co-cultured T cell hybrids. Thus the G9 clones do not appear to be recognizing known K^d motifs within these proteins. However, it may be that unconventional K^d binding peptides exist within proteins such as pre-proinsulin, and these can perhaps be determined using a peptide scan technique.

Other known β-cell autoantigens were previously tested, in the form of peptides, for their antigenicity to the G9 T cell clones, using IL-2 proliferation assays and cytotoxic ^{51}Cr-release assays. GAD65 and GAD67 were tested and not found to induce T cell activation. The antigenicity of insulin, proinsulin, and preproinsulin were additionally tested using these assays, as well as by directly adding peptides to the supernanent of K^d-COS cells co-cultured with BW/G9 T cell hybrids, and applying the *lacZ* assay. The peptides did not activate the T cell clones or hybrids, and thus do not appear to be the ligands recognized by these CD8 T cell clones.

Discussion

1. Islet cell cDNA library

Expression cloning of antigenic peptides, as an alternative to biochemical purification, is not dependent upon the total amount of obtainable protein, but requires the extraction of relatively large amounts of highly purified mRNA from the appropriate tissue, for the cDNA library construction. Thus, the availability of high quality starting material is critical for successful expression cloning strategies. The construction of this islet cDNA library used mRNA purified from the islets of 200 non-diabetic male NOD mice. The library is an unamplified library prepared from stringently size-selected cDNA (Edge Biosystems, Maryland). Although this cDNA library has been skillfully constructed from excellent starting materials, it can be that target antigens are resistant to cloning, as when they are too large (ie. > 5 kb in size), or too small. The cut-off size of the islet cell cDNA library used in this study is 700 base pairs. Thus the cDNAs corresponding to very small proteins could be excluded from this library.

2. MHC class I expressing COS cells and the *lacZ* expression cloning strategy.

In this thesis we are applying a newly described expression cloning strategy in an effort to identify the antigen recognized by the diabetogenic CD8 T cell clone. Here we show that COS-7 cells can stably express the NOD mouse K^d MHC class I allele at high levels (figure #). Our prediction that COS cells could function as APCs for NOD mouse K^d-restricted T cells was based on previous findings that the mouse K^b Class I molecule can interact appropriately with the simian antigen processing machinery existing in COS

cells (Karttunen et al, 1992). It was determined that OVA expressing K^b-COS cells activated a murine OVA/K^b-specific T cell, and that the peptide generated by the K^b-COS cell was identical to the peptide generated by OVA expressing mouse cells. Furthermore, studies of antigen-MHC ligands have not revealed any instances of species specific antigen processing and peptide formation, suggesting that the antigen processing machinery is highly conserved amongst mammals (Falk et al., 1991; Karttunen et al., 1992). Therefore, it seems likely that we are using an expression cloning system which generates peptide-K^d ligands on transfected K^d-COS cells that would be identical to the peptides generated in similarly transfected K^d expressing mouse cells. Here we are assuming that the presence of the appropriately restricted MHC molecule is sufficient for the appropriate generation of peptide-K^d ligands.

Despite the compatibility seen between the murine K^d MHC class I molecule and the COS cell's simian antigen processing machinery, it is important to consider the possibility that certain peptides may be resistant to processing and presentation of the K^d Class I pathway by COS cells. In order to demonstrate that K^d class I molecules process and present transfected cDNA appropriately in COS cells, we will determine whether K^d-restricted CD8 T cells, specific for the *listeria* P60 protein, are activated when co-cultured with K^d-COS cells transiently transfected with the P60 gene (provided by E. Pamer, Yale University School of Medicine). This model does not however directly test the ability of the G9 T cell clone to bind and become activated by peptide-K^d ligands on COS cells, and we would need to assume that our clone's TCR functions identically to that of the P60-K^d specific clone.

This P60-K^d model will not only determine if the mouse K^d Class I molecule interacts appropriately with the COS cell's antigen processing

machinery, but will also serve as a marker for successful transfections during the islet library screening process. The timely screening of 10^7 clones in the islet library required the semi-synchronous transfection of many cDNA pools. As a result COS cells were not consistently exposed to the Superfect reagent for the same length of time. Over-exposure to the Superfect reagent is damaging to cells. Therefore some pools may have been transfected for sub-optimal amounts of time, whereas others may have been transfected for too long, and therefore damaged. This variation in transfection efficiency could be monitored using the P60-K^d-specific T cells and P60 expressing K^d-COS, and the most efficient transfection time-span could be determined.

3.*LacZ*-inducible BW/G9 T cell hybrids

A. Increasing the sensitivity of the lacZ assay

As an alternative to conventional T cell activation assays, the particular strategy used here takes advantage of an NFAT-*lacZ* reporter which has been incorporated into the BW5147 fusion partner. BW cells were fused to the diabetogenic CD8 T cell clone, G9, to generate *lacZ*-inducible BW/G9 T cell hybrids. Here we demonstrate that when activated by NOD mouse islets, BW/G9 hybrids express the *lacZ* gene, and turn dark blue in the presence X-gal (figure 7). As such, these hybrids have been used as a single T cell activation assay to probe for ligand expression on APCs. Previously, this assay has been shown in the model system using ovalbumin, to be sufficiently sensitive to allow the detection of OVA cDNA at a frequency of $1:10^3$ - 10^4 (Karttunen et al., 1992). Here we used *lacZ*-indicator BW/G9 to probe for antigenic cDNAs in large pool sizes of 5×10^5 cDNAs. Assuming that the BW/G9 hybrids are as sensitive as the OVA specific B3Z indicator cells, and that the autoantigenic cDNA is abundant in the library, then our primary

screen should detect the peptide-ligand within these pools. However, at this point we have screened the entire library divided into 60 such pools, and no potentially positive signal has led to the identification of an antigenic cDNA. This represents an inherent difficulty that can be encountered with expression cloning strategies, where the target mRNA is rare and poorly represented in the islet cell library, and thus remains undetected by indicator T cells during the primary screening of very large pools of cDNA.

This difficulty can be addressed in two ways. First, the library screening can be carried out using smaller pools of cDNA, and we have begun the process of subdividing large pools into ten-fold smaller (10^4 cDNA) pools of cDNA. This will increase the proportion of candidate cDNAs within each pool, bringing their frequency into a range that the T cell hybrids are able to detect. However, this also creates a ten-fold increase in the number of pools to be screened, significantly increasing the time and effort involved in screening the library. The second approach is to increase the sensitivity of the BW/G9 hybrids. Since we do not know the antigen-specificity of the T cell hybrids (that is what we are determining), we cannot directly measure their sensitivity. Yet, it is possible to evaluate the BW/G9 hybrids' general reactivity through their co-culture with target tissue (ie. pancreatic islets), assuming that the T cell ligand is expressed at consistent densities on islet cells. It can be expected that the more sensitive the hybrids are the more will turn blue in the presence of islets. Recently, findings indicate that the efficiency of the NFAT-*lacZ* reporter construct varies depending on where it is incorporated into the indicator cell's genome (J. Karttunen, personal communication). The BW hybrid partner used here already contained the reporter construct in the DNA at the time it was fused to the G9 clone. Thus, the most highly islet-reactive BW/G9 hybrids were selected for, but with the

lacZ reporter fixed in one position. Thus, an approach to increasing the sensitivity of the indicator T cell hybrids is to first fuse the G9 clone to the BW fusion partner, without the lacZ reporter, and select for the BW/G9 hybrids most highly activated by islets. Following this, the NFAT-*lacZ* reporter construct can be transfected into these hybrids, where once again the most highly *lacZ*-expressing cells are selected for.

B. Pursuing a potentially positive signal by limiting dilution

By far the biggest undertaking in the screening of a library is the task of identifying a primary pool of cDNA which contains the sought after antigen. This is because many factors, such as the abundance of autoantigen cDNA, and the sensitivity of indicator T cells, which are contingent on the feasibility of the expression cloning strategy, are unknown. However, once a positive pool is identified, the process of limiting dilution should facilitate the further identification of positive, sub-divided pools. This is because the candidate cDNAs are more highly represented in successive subdivisions, and should yield increased levels of *lacZ* activity in indicator T cells. In fact, it has been suggested that soon after the enrichment of a pool containing antigenic cDNAs, a positive signal could exponentially increase from a rare cluster of blue cells to the generation of many *lacZ*-positive wells, similar to those seen with islet-activated hybrids (Karttunen et al., 1992). However, this is not clear with our BW/G9 hybrids. We did not observe this phenomenon when a potentially positive pool, which had yielded a small cluster of blue cells, was pursued by limiting dilution. The *lacZ* activities remained only slightly greater (about 10 fold more blue cells) than background levels on subsequent sub-divisions. It may be that this was a false positive signal. On the other hand, attaining large increases in a signal may be possible only when the

antigen is expressed at levels found *in vivo*. This level may be very high, and may be reached only using small pool sizes at the end of the dilution process. For these reasons, we pursued this potentially positive signal until individual cDNAs were isolated and tested for their antigenicity.

Pursuing a potentially positive signal in the absence of further feedback information (ie. confirming that the signal is real) risks investing a tremendous amount of time and effort into the isolation of candidate cDNAs which are not the true autoantigens recognized by the indicator T cell. Therefore, it would be extremely useful to have a second equally sensitive assay, which can be used concomitantly with the *lacZ* assay, to substantiate a potential positive signal. For instance, the G9 T cell clone itself is very sensitive, and proliferates efficiently in the presence of islets. The clones could perhaps confirm a positive *lacZ* assay by using proliferation and/or cytotoxic assays with the pool in question. It must be noted, however, that as with T cell hybrids, these clones can proliferate non-specifically, generating background signals which are difficult to distinguish from weakly positive signals. Alternatively, Boon and colleagues have successfully identified the antigens of several tumor-specific CD8 T cells (Boon et al., 1989). They have done so using an expression cloning strategy where the library was screened in stably transfected APCs and tested for their ability to stimulate tumor necrosis factor (TNF) release by cytotoxic CD8 T cells (Traversari et al., 1992; van der Bruggen et al., 1991). Though stable transfections, as compared to transient transfections, take longer to establish, such an approach may prove useful as a second assay to confirm positive signals generated by the *lacZ* assay.

C. Stability of lacZ-inducible BW/G9 hybrids

It was observed that after several weeks in culture BW/G9 T cell hybrids would gradually lose their islet-cell reactivity. The BW fusion partner of these hybrids is an immortalized cell which allows the hybridomas to multiply indefinitely. As a result, T cell hybrids tend to proliferate extremely rapidly, and undergo many cell divisions over short periods of time. As with many tumor cell lines, T cell hybrids are inherently unstable cells, and over time tend to lose essential surface molecules including their TCRs, CD8, and CD3 molecules. During the screening, it was also observed that as with the positive controls, hybrids had less background *LacZ* activity over time. Having decreased non-specific background fortunately compensated for the fact that the T cell hybrids also became less sensitive with time. Furthermore, as a means of maintaining islet-reactive hybrids in culture, frozen stocks of new hybrids were thawed to replace old hybrids every fourth week. Nonetheless, the gradual loss of T cell reactivity from one weekly set of transfections to the next does add some degree of variation to the sensitivity of our T cell probe. Since the feasibility of expression cloning requires that the *lacZ* assay be sensitive enough to detect antigen-expressing APCs at low frequencies, it would be advantageous to use indicator cells that did not tend to lose their reactivity to antigen. One option is to incorporate the *lacZ* reporter construct directly into the T cell clones, which proliferate indefinitely when cultured with islets, and have no propensity to lose their sensitivity to antigen over time. These highly responsive clones could then be used to screen for their autoantigen using a *lacZ* assay. The successful development of such indicator T cells would however be limited by the fact that T cell clones are notoriously difficult to permanently transfect (S. Wong, personal communication).

4. Nature of the autoantigenic target of CD8 T cells, cloned from young NOD mice.

A. Confirmation of peptide antigenicity

Following the isolation of candidate cDNAs by limiting dilution, they will be sequenced in order to identify the nature of the antigen. These sequences can then be used to isolate and characterize the genomic DNA sequences of these genes. As described in the results section, follow-up experiments with these candidate antigens can then done in order to establish their antigenicity. Identifying the cDNA and thus the amino acid sequence of candidate antigens will allow the construction of the minimal peptide determinants within the autoantigen sequence, based on the motifs specific for the K^d reported by Ramensee and colleagues (Falk et al., 1991). This will allow the identification of the peptide antigen in a functional assay and reduces the amount of screening of a possibly much larger protein sequence. However, if the computer-directed synthesis of K^d motifs does not identify antigenic peptides within candidate genes, we will consider examining these sequences using peptide scans. Peptide scan technology is available in our lab, and entails synthesizing and screening peptides, about 8-10 amino acids in length, within a given protein. This approach may well turn up an unconventional K^d binding peptide perhaps in the pre-proinsulin sequence. The identification of the CD8 T cell autoantigen will also provide a different, and presumably much more efficient, method of growing the cloned T cell lines. This could potentially simplify the isolation and propagation of T cell clones without the continuous need for islets as a source of antigen.

B. Diversification of the T cell autoimmune response

It will be important to determine whether or not all of the cloned T cell lines derived from young NOD islets (Wong et al., 1996) are responsive to the same autoantigen. This question can also be applied to the lab's previously isolated T cell clones (Reich et al., 1989). These were isolated from mice at a much more advanced stage of the disease process. This would support the hypothesis that in diabetes, as with other experimental autoimmune diseases, the initial disease stages involves a T cell response to a very specific antigen on β cells. It has been established that both autoreactive T cells and T-cell-dependent autoantibodies that characterize organ-specific autoimmune diseases recognize several distinct molecules, most of which are restricted to the target cells. This suggests that intermolecular epitope spreading takes place during autoimmune responses. In addition, the recent observation that a very limited number of Vβ-genes and VDJ-sequences are possibly used by T cells isolated from single islets, suggests that pathogenic T cell infiltrates originate from a limited number of T cell clones (Sarukhan et al., 1995). Thus, a diversity of CD8 T cells, derived from older mice, would be consistent with the diversification of the T cell response as diabetes progresses.

C. Islet-specificity of the CD8 T cell autoantigen

Once a candidate cDNA is identified as being antigenic, further investigation into the islet-specificity of this antigen will be carried out. Since the peptide antigen is presented in the context of MHC class I, a molecule which is expressed ubiquitously in other tissues, it will be interesting to determine whether the identified autoantigen is expressed in and presented by other tissues. Furthermore, it will be determined whether the autoantigen is presented on the islets of other non-NOD mice which express the same or

different MHC class I molecule. In principle this has been demonstrated where the diabetogenicity of the CD8 clones is preserved when transferred to H-2K^d expressing CB17.scid mice, but not B6.scid, which express H-2K^b (Wong et al., 1996).

Interestingly, when several candidate cDNAs were sequenced and identified, one was identified as the β cell specific molecule, preproinsulin, whereas two others were identified as preproglucon, synthesized by islet α cells, and α-amylase, produced by the exocrine pancreas. Although none of these proteins appear to be the antigen recognized by these cloned CD8 T cells, their diversity raises the interesting question of whether the CD8 T cells recognize an antigen expressed only on the islet β cells, or if they can be triggered by antigen-expressing islet α cells, or even the exocrine pancreas, before going on to destroy β cells. It is possible that this would take place if the inciting cell, expressing B7 co-stimulatory molecules, activated reactive T cells and led to the expression of FasL on the T cell (Alderson et al., 1995; Russell et al., 1993). When these FasL-expressing activated T cells encounter β cells which bear the Fas receptor on its surface, β cells could undergo apoptosis (Chervonsky et al., 1997). On the other hand, it has recently been demonstrated that class I-restricted CD8 T cells can be stimulated by human dendritic cells, which efficiently present antigen acquired from self-tissue (Albert et al., 1998). This suggests that cytotoxic T cells are not restricted to recognizing antigens that are solely synthesized in the cytoplasm of their target cells.

Nonetheless, there exists strong evidence that the antigenic targets of cytotoxic T cells are expressed on the islet β cells themselves. All of the autoantigens identified to date in IDDM are expressed on islet β cells. In fact insulin and proinsulin are expressed only by β cells. Furthermore, it has been

shown that the transfer of diabetogenic T cells into β-cell-depleted NOD mice (whose β cells have been selectively destroyed) does not allow the survival of such cells, as demonstrated by their inability to cause diabetes in a secondary transfer into prediabetic NOD mice bearing functional β cells (Larger et al., 1995). This suggests that islet β cells bear the target antigens of these diabetogenic T cells and drive the autoimmune response. Finally, type 1 diabetic patients show decreasing titers of β-cell specific autoantibodies which eventually disappear following the clinical onset of their disease, when all β cells are destroyed (Irvine et al., 1977). Similarly, these autoantibodies rapidly reappear following islet or pancreas transplantation (Sutherland et al., 1984).

5. Antigen-specific immunotherapy

A very important goal once, and if, an autoantigen is elucidated, is to explore the potential use of the antigen as a treatment modality for disease prevention. Autoantigen-based immunotherapy has previously been attempted by targeting the autoreactive T cells for immune intervention. One way to block their pathogenic activity is to induce tolerance in the autoreactive T cells by using autoantigen-derived peptides. This selectively eliminates the unwanted T cells. Here, it will be determined if the autoantigenic peptide can be manipulated such that it binds to T cell but does not induce a response, thus effectively antagonizing the T cell response. These antagonist peptides could be used in the NOD mouse *in vivo* to prevent disease. The autoantigenic peptide will be mutated to generate variant peptides, which can then be characterized *in vitro* using agonist peptides and the cloned cells. T cell hybrids will also be used to test for antagonizing effects of these peptides. Once antagonist peptides are identified *in vitro*, they will then be injected into mice and tested for their *in vivo*

ability to inhibit diabetes. Another antigen-specific approach to inhibit the activity of the diabetogenic T cells is through oral and nasal administration of antigen or antigenic peptides. Such immunotherapy was carried out using GAD and insulin, and resulted in the down-regulation of self-reactive T cells, and in the case of insulin, the production of protective gamma-delta (γδ) T cells (Harrison et al., 1996; Tian et al., 1996; Zhang et al., 1991). Thus, oral tolerance may prove to be another method by which the CD8 T cell autoantigen can be used to treat and prevent disease.

Previous studies have shown that the treatment of young mice with the β-cell antigen GAD, can tolerize GAD-specific T cells and in turn prevent diabetes in the NOD mouse (Kaufman et al., 1993; Tisch et al., 1993). This protection seems to result from the induction of a protective Th2-like response (Zechel et al., 1997). Intrathymic administration of GAD, as a means of inducing a central tolerance (by deletion), has been less conclusive, and some authors have even found peptide administration to exacerbate the diabetic process rather than delay or suppress it (Cetkovic-Cvrlje et al., 1997). More recent studies have demonstrated tolerance induction to insulin (French et al., 1997; Muir et al., 1995; Zhang et al., 1991), and down-regulation of T cell reactivities to HSP60 protects NOD mice from diabetes (Birk et al., 1996; Elias and Cohen, 1994). In the case of multiple sclerosis (MS), treatment with immunodominant peptides of the myelin basic protein (MBP) can prevent and treat the CD4 T cell-mediated murine model of experimental autoimmune encephalomyelitis (Smilek et al., 1991; Wraith et al., 1989). Thus, it appears that peptide-specific immunotherapy, when applied to highly defined models of autoimmunity, can be effective.

In diseases such as IDDM, or MS, in which the target autoantigen(s) is not known and a number of autoantigens appear to be involved in the

disease process, it is less clear how such a peptide-specific approach could be feasible. At this time there is no consensus as to which antigens in IDDM are most effective in this process. However, studies do suggest that this form of immunotherapy may be successful in a clinical setting, where success hinges on whether the therapy can be used to treat an ongoing autoimmune response or whether it can be useful only in disease prevention (Tisch and McDevitt, 1994). Furthermore, there are many questions that remain unanswered. How many β-cell autoantigens have not been identified, and do primary or triggering antigens exist in NOD mice? How are NOD mouse autoantigens similar to or different from human β-cell antigens? In the past, most assays have focused on the antigens recognized by CD4 T cells. It is now becoming apparent that CD8 T cells play a critical role as initiators of insulitis. Thus, it is likely that by elucidating the autoantigens recognized by CD8 T cells, we will be able to address many of these unanswered questions.

Conclusion

In this thesis we describe the application of the *lacZ* assay as a means of identifying the target antigen of a CD8 T cell clone, isolated from young NOD mice, which transfers rapid-onset diabetes in the absence of CD4 T cells. Defining the ligands of T cells involved in IDDM is an extremely important component of understanding the immunological mechanisms underlying diabetogenesis and autoimmunity in general. CD8 T cells appear to be critical initiators of diabetes, and it may well be that the antigenic targets of CD8 T cells play a role in disease induction. However, the antigenic targets of CD8 T cell have not been identified in type 1 diabetes. Their identification may provide insight into the existence and nature of a primordial autoantigen in the pathogenesis of IDDM. Identifying T cell autoantigens has been a difficult task. Here we make use of an expression cloning strategy which takes advantage of a highly sensitive single-cell T cell assay. We aim to manipulate the *lacZ* assay in an effort to further increase the sensitivity of the indicator T cells, and continue to screen the islet cDNA library in smaller pools. This will help to enable our endeavor to identify the autoantigen of the diabetogenic CD8 T cell clone, and subsequently explore its potential as a treatment modality for disease prevention.

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